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PROVISIONAL APPLICATION COVER SHEET

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Docket Number	GC839P	Type a plus sign (+) inside this box →	+
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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

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Respectfully submitted,

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PROVISIONAL APPLICATION FILING ONLY

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Date: April 15, 2004

By: 
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**PROVISIONAL PATENT
ATTORNEY DOCKET NO. GC839P**

CAB MOLECULES

FIELD OF THE INVENTION

- The present invention relates to CAB molecules, ADEPT constructs directed against
- 5 CEA and their use in therapy.

BACKGROUND

- Traditional therapeutic molecules circulate freely throughout the body of patients until they are removed from circulation by the liver or another mechanism of clearance.
- 10 Such non-targeted molecules can exert pharmacological effects indiscriminately on a wide range of cells and tissues. Indiscriminate targeting can cause serious side effects in a patient. The problem may be particularly acute when the molecule is highly toxic (e.g., in the case of a chemotherapeutic agent where the therapeutic window, the difference between an efficacious and injurious or even lethal dose, can be small).
- 15 In recent years, researchers have attempted to develop compounds that specifically affect particular groups of cells, tissues or organs. Most of the compounds target a particular tissue by preferentially binding a particular target molecule displayed by the tissue. By preferentially affecting targeted cells, tissues or organs, the therapeutic window can be increased, which in turn increases the opportunity for a successful treatment regimen
- 20 and/or reduces the occurrence of side effects.
- Preferential binding is employed in antibody-directed enzyme prodrug therapy (ADEPT). *See, e.g., Xu et al.*, 2001, Clin Cancer Res. 7:3314-24.; Denny, 2001, Eur J Med Chem. 36:577-95. In ADEPT, an antibody or antibody fragment is linked to an enzyme capable of converting an inactive pro-drug into an active cytotoxic agent. An ADEPT
- 25 conjugate is administered to a patient, and the conjugate is localized to a target tissue via antibody/antigen binding. The prodrug is subsequently administered, and the prodrug circulates throughout the patient's body but causes few or no side effects because the

prodrug is in the inactive form and is activated by the ADEPT antibody-enzyme conjugate only in the vicinity of the target tissue. Thus, a relatively low concentration of active drug is present throughout the body, but a relatively high concentration of active drug is produced in the vicinity of the target tissue, increasing the therapeutic window of the toxin at the desired site.

In ADEPT, the antibody or antibody portion of a construct binds to an antigen to achieve localization, so selecting the proper antigen is important (e.g., an antigen that has a high tumour/normal expression profile). An antigen of particular interest frequently found on the cell surface in cancer tissues is carcinoembryonic antigen (CEA). CEA was first described by Gold and Freedman, *J. Exp. Med.*, 121, 439-462, (1965). CEA is highly expressed in tumor tissue and also found at a lower concentration in some normal organs, particularly in the digestive tract.

Many antibodies to tumor antigens cross-react with related antigens. Systemic application of a MAb that is cross-reactive with a related antigen must be avoided to preclude risk of potentially severe side effects. Accordingly, the development of antigen-specific monoclonal antibodies for in vitro and in vivo diagnosis and therapy requires a good knowledge of the number, quality and biodistribution of related cross-reactive antigens. Careful immunochemical characterization of the MAb to be used is required with respect to its specificity and affinity for the target antigen and for related antigens.

Murine MAb T84.66 (ATCC Accession No. BH 8747) IgG1,k shows a high affinity constant to CEA and no cross reactivity to other members of the CEA gene family.

A significant potential side effect of ADEPT therapy is the development of antibodies against the targeted enzyme during therapy. The production of human anti-mouse antibodies (HAMA) leads to reduced efficiency of the MAb and to potentially serious manifestations of acute and chronic allergic complications for the patient. See Levy, et al. *Ann. Rev. Med.* 34:107-116 (1983); Houghton, et al. *Proc. Natl. Acad. Sci. USA*, 82:1242-1246 (1985) and Sears, et al. *J. Biol. Resp. Modifiers* 3:138-150 (1984). Antibody formation has been observed during a clinical trial of a CEA-directed antibody-enzyme conjugate two weeks after treatment, which prevented subsequent rounds of treatment [Napier, M. P., S. K. Sharma, C. J. Springer, K. D. Bagshawe, A. J. Green, J. Martin, S. M. Stribbling, N. Cushen, D. O'Malley and R. H. Begent (2000) *Clin Cancer Res* 6, 765-72,

Antibody-directed enzyme prodrug therapy: efficacy and mechanism of action in colorectal carcinoma].

The risk of eliciting an immune reaction is high for microbial proteins. The use of human enzymes for ADEPT has been investigated in pre-clinical studies [Smith, G. K., S. Banks, T. A. Blumenkopf, M. Cory, J. Humphreys, R. M. Laethem, J. Miller, C. P. Moxham, R. Mullin, P. H. Ray, L. M. Walton and L. A. Wolfe, 3rd (1997) *J Biol Chem* 272, 15804-16, Toward antibody-directed enzyme prodrug therapy with the T268G mutant of human carboxypeptidase A1 and novel in vivo stable prodrugs of methotrexate].

Although the risk of antibody formation can be reduced for human protein as compared to microbial protein, human proteins can also elicit immune reactions when administered to people. The consequences of eliciting an immune reaction against a human protein can be very significant, as such a treatment could trigger an auto-immune disease.

The risk of eliciting an immune reaction may be great for an ADEPT construct that contains at least two two potential risks: the antibody portion and the enzyme portion.

SUMMARY OF THE INVENTION

The present invention relates to CAB molecules, ADEPT constructs directed against CEA and their use in therapy, especially with prodrugs as described herein. The molecules of the current invention have been preferably deimmunized and do not elicit an immune response and can be produced in high yield.

In a first aspect, the CAB molecule comprises an antibody/enzyme conjugate, wherein the antibody portion binds to CEA. In a preferred embodiment, the enzyme comprises a beta-lactamase.

In a preferred embodiment, the CAB molecule has an unmodified amino acid sequence. In a preferred embodiment, the CAB molecule has an amino acid sequence modified from the amino acid sequence set forth in SEQ ID NO:2, and the modification is located at at least one of positions 12, 72, 283 or 586, wherein position numbering is with respect to SEQ ID NO:2 as shown in Figure 4. In a preferred embodiment, the CAB molecule has both of the following modifications: 12 and 72. In a preferred embodiment, the CAB molecule has all of the following modifications: 12, 72, 283 and 586.

In a preferred embodiment, the CAB molecule has at least one of the following modifications: A12S, R72G, K283A or S586A, wherein position numbering is with respect to SEQ ID NO:2 as shown in Figure 4. In a preferred embodiment, the CAB molecule comprises a CAB1.11 molecule, the CAB1.11 molecule comprising the following
 5 modifications: A12S and R72G. In a preferred embodiment, the CAB molecule comprises a CAB1.11i molecule, the CAB1.11i molecule comprising the following modifications: A12S, R72G, K283A and S586A.

In a preferred embodiment, the CAB molecule comprises CAB 1.10 having SEQ ID NO:2, CAB1.11 having SEQ ID NO:7 or CAB1.11i having SEQ ID NO:8.

10 In a second aspect, the invention is drawn to a nucleic acid encoding a CAB molecule as set forth herein. In a third aspect, the invention is drawn to treating a subject in need thereof, comprising administering to the subject a CAB molecule, as provided herein, and a prodrug that is a substrate of the CAB molecule. In a fourth aspect, the invention is drawn to a pharmaceutical composition comprising a CAB molecule.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 sets forth unmodified sequences disclosed in the invention. Figure 1A (SEQ ID NO:1) sets forth the amino acid sequence of the T84.66 antibody as described in Shively et al. and as disclosed in the invention provided herein; Figure 1B sets forth the
 20 nucleotide sequence of the T84.66-derived antibody (SEQ ID NO:3); Figure 1C sets forth the amino acid sequence for BLA (SEQ ID NO: 11); Figure 1D sets forth the nucleotide sequence for BLA (SEQ ID NO:12); Figure 1E sets forth the amino acid sequence for the 1.10 (SEQ ID NO: 2) construct that is T84.66 fused to BLA and includes a linker; Figure 1F sets forth the nucleotide sequence for the 1.10 construct (SEQ ID NO:4). Underlining
 25 indicates the scFv portion of the molecule, and italics indicate the linker between the vl and vh portions of the scFv.

Figure 2 sets forth the modified CAB 1.11 antibody portion of the current invention. Figure 2A sets forth the amino acid sequence that has been modified and comprises a CAB 1.11 antibody portion (SEQ ID NO:5); Figure 2B sets forth the nucleotide sequence
 30 sequence (SEQ ID NO:6) that has been modified and comprises a CAB1.11 antibody portion.

Figure 3 sets forth amino acid sequence of the deimmunized BLA portion (SEQ ID NO:13). Mutations from unmodified BLA (provided in Figure 1) are shown in bold; numbering has been retained to remain consistent with the CAB1.11i construct.

Figure 4 sets forth sequences for the CAB1.11 and CAB1.11i molecules. Figure 4A sets forth the amino acid sequence of the CAB1.11 molecule (SEQ ID NO:7), which includes an antibody portion modified to improve expression, as described in the Examples, and also includes the BLA portion; Figure 4B sets forth the nucleotide sequence of the CAB1.11 molecule (SEQ ID NO:9); Figure 4C sets forth the amino acid sequence of the CAB1.11i molecule (SEQ ID NO:8), which includes the deimmunized BLA portion, as set forth in the Examples; Figure 4D sets forth the nucleotide sequence that encodes the CAB1.11i molecule (SEQ ID NO:10), Figure 4E sets forth the nucleotide sequence that encodes the plasmid, pHR19.2. In the Figure showing the CAB1.11i molecule, the scFv portion of the molecule has been underlined. The four mutations described in the Examples herein have been underlined. The linkers provided herein have been italicized. Two of the mutations are in the scFv portion of the molecule, and two of the mutations are in the BLA portion of the molecule.

Figure 5 shows the plasmid map for pHR19.2.

Figure 6 sets forth the binding of variants to target CEA. The x-axis shows the variant designation, as described herein, and the y-axis shows the % specific binding.

Figure 7 shows a graph setting forth the results from the fermentation runs of EB101.1/pHR19.2 as set forth in Example 6. The x-axis shows EFT measured in hours, and the y-axis shows lactamase activity, measured in mg/L.

Figure 8 sets forth the purification process for CAB1.11i, as described in Example 7.

Figure 9 shows an SDS PAGE electrophoresis of CAB 1.11i. Lane 1 shows a molecular weight standard, lanes 3-5 show unrelated proteins and lane 6 shows 1.11i.

Figure 10 shows binding and off-rate curves for CAB1.11i. Figure 10A shows a binding curve, the x-axis showing amount, in nM, of CAB1.11i bound, and the y-axis shows how much CAB1.11i was added, again shown in nM. Figure 10B shows off-rate, as described in Example 9, with time measured in minutes on the y-axis and percent BLA activity bound shown on the x-axis.

Figure 11 shows CD, as set forth in Example 10.

Figure 12 is a graph demonstrating tissue localization of CAB 1.11i. The x-axis shows time after administration, in hours, of the CAB 1.11i construct. The y-axis shows concentration in ug/g, of the CAB construct in different organs, as indicated by color, as shown in the chart at the right. The graph shows that CAB1.11i was eliminated rapidly from plasma, liver and kidney and localized to tumour.

Figure 13 shows the tumour to blood ratio of CAB 1.11i. The x-axis shows time, in hours, after administration of the CAB 1.11i construct. The y-axis shows the level of tumour to blood. High tumor to blood ratios were achieved and sustained.

Figure 14 shows demonstrated activity in a human colorectal cancer xenograft mouse model. The x-axis shows day number. The y-axis shows tumour volume measured in cubic millimeters. See, for example, Example 12.

DETAILED DESCRIPTION OF THE INVENTION

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described. For purposes of the present invention, the following terms are used as described below.

"CAB" molecule shall mean a targeted agent that binds to a carcinoembryonic antigen (CEA) target or microtarget and an enzyme, such as BLA. A CAB molecule may have an unmodified sequence or a modified sequence, wherein the unmodified sequence comprises the amino acid sequence set forth in SEQ ID NO:2. SEQ ID NO:2 sets forth a CAB molecule that includes BLA as shown in Figure 4. Position numbering as described in this document is with respect to SEQ ID NO:2, as set forth Figure 4.

A "modified" sequence refers to a sequence that includes at least one mutation.

An "unmodified" sequence, as set forth herein, refers to a sequence that has not been modified and, thus, does not include at least one mutation, as set forth herein.

Examples of unmodified sequences of the invention include, but may not be limited to, T84.66 (SEQ ID NO:1), CAB 1.10 (SEQ ID NO:2) and BLA (SEQ ID NO:11).

Unmodified sequences may be modified, as described herein, to produce preferred embodiments of the invention.

A "targeted agent" is a chemical entity that binds selectively to a microtarget of interest. Examples of targeted agents are antibodies, peptides and inhibitors. Of interest
5 are targeted enzymes that have a desired catalytic activity and that can bind to one or more target structures with high affinity and selectivity. Targeted enzymes retain at least most of their activity while bound to a target.

A "binding moiety" is a part of a targeted agent (or an ADEPT construct, e.g., CAB molecule) that binds a microtarget. A binding moiety can comprise more than one region,
10 either contiguous or non-contiguous, of a CAB.

An "active moiety" is a part of a targeted agent (or an ADEPT construct, e.g., CAB molecule) that confers functionality to the agent. An active moiety can comprise more than one region, either contiguous or non-contiguous, of, for example, a CAB molecule. In particular, an active moiety can be a beta-lactamase.

15 The term "protein" is used interchangeably here with the terms "peptide" and "polypeptide," and refers to a molecule comprising two or more amino acid residues joined by a peptide bond.

The terms "cell", "cell line", and "cell culture" can be used interchangeably and all such designations include progeny. The words "transformants" or "transformed
20 cells" include the primary transformed cell and cultures derived from that cell without regard to the number of transfers. All progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same functionality as screened for in the originally transformed cell are included in the definition of transformants. The cells can be prokaryotic or eukaryotic.

25 The term "oligonucleotide" as used herein is defined as a molecule comprised of two or more deoxyribonucleotides or ribonucleotides. The exact size will depend on many factors, which in turn depend on the ultimate function or use of the oligonucleotide. Oligonucleotides can be prepared by any suitable method, including, for example, cloning and restriction of appropriate sequences and direct chemical synthesis by a method such as
30 the phosphotriester method of Narang et al., 1979, Meth. Enzymol. 68:90-99; the phosphodiester method of Brown et al., 1979, Meth. Enzymol. 68:109-151; the

diethylphosphoramidite method of Beaucage et al., 1981, Tetrahedron Lett. 22:1859-1862; and the solid support method of U.S. Pat. No. 4,458,066, each incorporated herein by reference. A review of synthesis methods is provided in Goodchild, 1990, Bioconjugate Chemistry 1(3):165-187, incorporated herein by reference.

5 The term "primer" as used herein refers to an oligonucleotide capable of acting as a point of initiation of synthesis when placed under conditions in which primer extension is initiated. Synthesis of a primer extension product that is complementary to a nucleic acid strand is initiated in the presence of the requisite four different nucleoside triphosphates and a DNA polymerase in an appropriate buffer at a suitable temperature. A "buffer" includes a
10 buffer, cofactors (such as divalent metal ions) and salt (to provide the appropriate ionic strength), adjusted to the desired pH.

A primer that hybridizes to the non-coding strand of a gene sequence (equivalently, is a subsequence of the noncoding strand) is referred to herein as an "upstream" or "forward" primer. A primer that hybridizes to the coding strand of a gene sequence is
15 referred to herein as a "downstream" or "reverse" primer.

Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, asparagine, glutamine, serine, threonine, tyrosine), nonpolar side chains (*e.g.*, alanine,
20 valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan, cysteine, glycine), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine). Standard three-letter or one-letter amino acid abbreviations are used herein. Equivalent substitutions may be included within the scope of the claims.

25 The peptides, polypeptides and proteins of the invention can comprise one or more non-classical amino acids. Non-classical amino acids include but are not limited to the D-isomers of the common amino acids, α -amino isobutyric acid, 4-aminobutyric acid (4-Abu), 2-aminobutyric acid (2- Abu), 6-amino hexanoic acid (Ahx), 2-amino isobutyric acid (2-Aib), 3-amino propionic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosine,
30 citrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, β -

alanine, fluoro-amino acids, designer amino acids such as β -methyl amino acids, α -methyl amino acids, α -methyl amino acids, and amino acid analogs in general.

The term "Ab" or "antibody" refers to polyclonal and monoclonal antibodies (MAb), chimeric antibodies, humanized antibodies, human antibodies, immunoglobulins or antibody or functional fragments of an antibody that binds to a target antigen. Examples of such functional entities include complete antibody molecules, antibody fragments, such as Fv, single chain Fv, complementarity determining regions (CDRs), V_L (light chain variable region), V_H (heavy chain variable region) and any combination of those or any other functional portion of an immunoglobulin peptide capable of binding to target antigen. In Example 1, the construct has the following order: vL-(GGGS)₆-vH; however, the example is non-limiting, and all orders of vL and vH, are contemplated to be within the scope of the invention. Furthermore, the length of the linker need not be 30 amino acids in length, as disclosed herein, and different linker lengths are contemplated to be within the scope of the invention.

The term "prodrug" refers to a compound that is converted via one or more enzymatically-catalyzed or physiologically-catalyzed steps into an active compound that has an increased pharmacological activity relative to the prodrug. A prodrug can comprise a pro-part or inactive moiety and a drug or active drug or detectable moiety. Optionally, the prodrug also contains a linker. For example, the prodrug can be cleaved by an enzyme to release an active drug. Alternatively, an enzyme could alter the prodrug to release a detectable moiety. In a more specific example, prodrug cleavage by the targeted enzyme releases the active drug into the vicinity of the target bound to the targeted enzyme. "Pro-part" and "inactive moiety" refer to the inactive portion of the prodrug after it has been converted. For example, if a prodrug comprises a PEG molecule linked by a peptide to an active drug, the pro-part is the PEG moiety with or without a portion of the peptide linker.

The term "drug" and "active drug" and "detectable moiety" refer to the active moieties of a prodrug. After cleavage of the prodrug by a targeted enzyme, the active drug acts therapeutically upon the targeted tumor, cell, infectious agent or other agent of disease. The detectable moiety acts as a diagnostic tool, and such detectable moieties are intended to be within the scope of the claims. The active drug can be any chemical entity that is able to

kill a cell, inhibit cell proliferation or act in concert with another drug to facilitate cell killing or inhibition of cell proliferation (e.g., drugs that predispose cells to apoptosis).

The term "% sequence homology" is used interchangeably herein with the terms "% homology," "% sequence identity" and "% identity" and refers to the level of amino acid sequence identity between two or more peptide sequences, when aligned using a sequence alignment program. For example, as used herein, 80% homology means the same thing as 80% sequence identity determined by a defined algorithm, and accordingly, a homologue of a given sequence has greater than 80% sequence identity over a length of the given sequence. Exemplary levels of sequence identity include, but are not limited to, 60, 70, 80, 85, 90, 95, 98 or 99% or more sequence identity to a given sequence.

Exemplary computer programs that can be used to determine identity between two sequences include, but are not limited to, the suite of BLAST programs, e.g., BLASTN, BLASTX, and TBLASTX, BLASTP and TBLASTN, which are well-known to one skilled and the art. *See also* Altschul *et al.*, 1990, *J. Mol. Biol.* 215: 403-10 and Altschul *et al.*, 1997, *Nucleic Acids Res.*, 25:3389-3402. Sequence searches are typically carried out using the BLASTP program when evaluating a given amino acid sequence relative to amino acid sequences in the GenBank Protein Sequences and other public databases. The BLASTX program is preferred for searching nucleic acid sequences that have been translated in all reading frames against amino acid sequences in the GenBank Protein Sequences and other public databases. Both BLASTP and BLASTX are run using default parameters of an open gap penalty of 11.0, and an extended gap penalty of 1.0, and utilize the BLOSUM-62 matrix. *See* Altschul, *et al.*, 1997.

A preferred alignment of selected sequences in order to determine "% identity" between two or more sequences, is performed using for example, the CLUSTAL-W program in MacVector version 6.5, operated with default parameters, including an open gap penalty of 10.0, an extended gap penalty of 0.1, and a BLOSUM 30 similarity matrix.

The present invention relates to CAB molecules, ADEPT constructs directed against CEA, and their use in therapy, especially with prodrugs as described herein. The molecules of the current invention have been preferably deimmunized and do not elicit an immune response.

In a first aspect, the CAB molecule comprises an antibody/enzyme conjugate, wherein the antibody portion binds to CEA. In a preferred embodiment, the enzyme comprises a beta-lactamase.

In a preferred embodiment, the CAB molecule has an unmodified amino acid sequence. In a preferred embodiment, the CAB molecule has an amino acid sequence modified from the amino acid sequence set forth in SEQ ID NO:2, and the modification is located at at least one of positions 12, 72, 283 or 586, wherein position numbering is with respect to SEQ ID NO:2 as shown in Figure 4. In a preferred embodiment, the CAB molecule has both of the following modifications: 12 and 72. In a preferred embodiment, the CAB molecule has all of the following modifications: 12, 72, 283 and 586.

In a preferred embodiment, the CAB molecule has at least one of the following modifications: A12S, R72G, K283A or S586A, wherein position numbering is with respect to SEQ ID NO:2 as shown in Figure 4. In a preferred embodiment, the CAB molecule comprises a CAB1.11 molecule, the CAB1.11 molecule comprising the following modifications: A12S and R72G. In a preferred embodiment, the CAB molecule comprises a CAB1.11i molecule, the CAB1.11i molecule comprising the following modifications: A12S, R72G, K283A and S586A.

In a preferred embodiment, the CAB molecule comprises CAB 1.10 having SEQ ID NO:2, CAB1.11 having SEQ ID NO:7 or CAB1.11i having SEQ ID NO:8.

In another embodiment, the CAB is an MDTA as described in PCT Application Number US03/18200, filed June 12, 2002 and incorporated herein by reference in its entirety. Some of the CAB molecules of the present invention have been shown to preferentially bind to a microtarget present on a target relative to binding of a non-target. The difference in binding can be caused by any difference between the target and non-target such as, for example, a difference in pH, oxygen pressure, concentration of solutes or analytes (*e.g.*, lactic acid, sugars or other organic or inorganic molecules), temperature, light or ionic strength. Preferential binding of the CABs of the current invention can be used to bind to a microtarget under a desired set of conditions, identify a target *in vitro*, *ex vivo*, *in situ* or *in vivo* (*e.g.*, a target tissue in a subject), kill a target cell or tissue, convert a prodrug into an active drug in or near a target tissue. It also can be used as a surface catalyst, for example, a targeted laccase. Other uses include, *e.g.*, targeted generation of a

compound (*e.g.*, H₂O₂ from glucose) and the targeted destruction of compounds (*e.g.*, a metabolite or signalling molecule from a particular tissue).

In one embodiment, the CAB is selected, made or modified using an affinity maturation method, *e.g.*, as described in PCT application US03/18187, with a priority date
5 filed June 12, 2002 and incorporated herein by reference in its entirety.

In another embodiment, the CAB is selected, made or modified using a loop-grafting method, *e.g.*, as described in U.S. Pat. App. Ser. No. 10/170,387, filed June 12, 2002 and incorporated herein by reference in its entirety.

In another embodiment, the CAB is a multifunctional polypeptide, *e.g.*, as described
10 in U.S. Pat. App. Ser. No. 10/170,729, filed June 12, 2002 and incorporated herein by reference in its entirety.

In another embodiment, the CABs of the invention are used for diagnostic or therapeutic applications such as those disclosed, for example, in United States patent 4,975,278, which is incorporated herein by reference in its entirety, as well as methods
15 well-known in the art.

In one embodiment, the CAB molecule further comprises an active moiety. The active moiety can be any molecule, or a part of a molecule, that has an activity. The activity can be any activity. Examples of types of activities that the active moiety can have include, for example, a detectable activity, an enzymatic activity, a therapeutic activity, a
20 diagnostic activity, a toxic activity or a binding activity. The active moiety can be a discrete part of the CAB, for example, an enzyme that is fused or conjugated to the binding moiety, or it can be an integral part of the CAB, for example, binding of the CAB to the microtarget can activate or inhibit an activity of the microtarget or the target, or the CAB can be a targeted enzyme of the type discussed below and in copending United States
25 Patent Application Serial Numbers 10/022,073 and 10/022,097, incorporated herein by reference in their entireties.

In another embodiment, the active moiety exhibits enzymatic activity, *e.g.*, it is an enzyme or an active fragment or derivative of an enzyme. Of particular interest are enzymes that can be used to activate a prodrug in a therapeutic setting. A large number of
30 enzymes with different catalytic modes of action have been used to activate prodrugs. *See, e.g.*, Melton & Knox Enzyme-prodrug strategies for cancer therapy (1999) and Bagshawe

et al., *Curr Opin Immunol* 11:579 (1999). Examples of types of enzymes that can be used to make the CABs of the present invention include, but are not limited to, proteases, carboxypeptidases, β -lactamases, asparaginases, oxidases, hydrolases, lyases, lipases, cellulases, amylases, aldolases, phosphatases, kinases, transferases, polymerases, nucleases, nucleotidases, laccases, reductases, and the like. See, e.g., co-pending U.S. Pat. App. Ser. No. 09/954,385, filed September 12, 2001, incorporated herein by reference in its entirety. As such, CABs of the invention can, for example, exhibit protease, carboxypeptidase, β -lactamase, asparaginase, oxidase, hydrolase, lyase, lipase, cellulase, amylase, aldolase, phosphatase, kinase, transferase, polymerase, nuclease, nucleotidase, laccase or reductase activity or the like. Examples of enzymes that can be used are those that can activate a prodrug, discussed below, and those that can produce a toxic agent from a metabolite, e.g., hydrogen peroxide from glucose. See Christofidou-Solomidou *et al.*, 2000, *Am J Physiol Lung Cell Mol Physiol* 278:L794.

In one embodiment, the present invention provides a CAB further comprising a β -lactamase ("BLA"). In another embodiment, the BLA is a targeted enzyme as described in co-pending United States Patent Application Serial Numbers 10/022,073 and 10/022,097, incorporated herein by reference in their entirety.

BLA enzymes are widely distributed in both gram-negative and gram-positive bacteria. BLA sequences are well known. A representative example of a BLA sequence is depicted in Figure 3. BLA enzymes vary in specificity, but have in common that they hydrolyze β -lactams, producing substituted β -amino acids. Thus, they confer resistance to antibiotics containing β -lactams. Because BLA enzymes are not endogenous to mammals, they are subject to minimal interference from inhibitors, enzyme substrates, or endogenous enzyme systems (unlike proteases), and therefore are particularly well-suited for therapeutic administration. BLA enzymes are further well-suited to the therapeutic methods of the present invention because of their small size (BLA from *E. cloacae* is a monomer of 39 kD; BLA from *E. coli* is a monomer of 30 kD) and because they have a high specific activity against their substrates and have optimal activity at 37° C. See Melton *et al.*, *Enzyme-Prodrug Strategies for Cancer Therapy*, Kluwer Academic/Plenum Publishers, New York (1999).

Examples of specific BLAs that can be used to make the CABs of the present invention include, but are not limited to, Class A, B, C or D β -lactamase, β -galactosidase, see Benito *et al.*, *FEMS Microbiol. Lett.* 123:107 (1994), fibronectin, glucose oxidase, glutathione S-transferase, see Napolitano *et al.*, *Chem. Biol.* 3:359 (1996) and tissue plasminogen activator, see Smith *et al.*, *J. Biol. Chem.* 270:30486 (1995). The β -lactamases have been divided into four classes based on their sequences. See Thomson *et al.*, 2000, *Microbes and Infection* 2:1225-35. The serine β -lactamases are subdivided into three classes: A (penicillinases), C (cephalosporinases) and D (oxacillinases). Class B β -lactamases are the zinc-containing or metallo β -lactamases. Any class of BLA can be utilized to generate a CAB of the invention.

In one embodiment of the invention, the BLA has a specific activity greater than about 0.01 U/pmol against nitrocefin using the assay described in United States Patent Application Serial Number 10/022,097. In another embodiment, the specific activity is greater than about 0.1 U/pmol. In another embodiment, the specific activity is greater than about 1 U/pmol. Preferably, these specific activities refer to the specific activity of the BLA when it is bound to a microtarget.

In one embodiment, the BLA enzyme in the CAB comprises the amino acid sequence set forth in SEQ ID NO:11. In another embodiment, the BLA enzyme in the CAB is at least 50%, 60%, 70%, 80%, 90%, 95%, 98% or 99% or more identical to the sequence depicted in Figure 4.

In a preferred embodiment, the CAB is CAB1.11 or CAB1.11i.

The targets bound by the CAB, or one or more binding moieties, can be any substance or composition to which a molecule can be made to bind to CEA. In one embodiment, the target is a surface. In one embodiment, the surface is a biological surface. In another embodiment, the biological surface is a surface of an organ. In another embodiment, the biological surface is a surface of a tissue. In another embodiment, the biological surface is a surface of a cell. In another embodiment, the biological surface is a surface of a diseased organ, tissue or cell. In another embodiment, the biological surface is a surface of a normal or healthy organ, tissue or cell. In another embodiment, the surface is a macromolecule in the interstitial space of a tissue. In another embodiment, the biological surface is the surface of a virus or pathogen. In another embodiment, the surface is a non-

biological surface. In another embodiment, the non-biological surface is a surface of a medical device. In another embodiment, the medical device is a therapeutic device. In another embodiment, the therapeutic device is an implanted therapeutic device. In another embodiment, the medical device is a diagnostic device. In another embodiment, the diagnostic device is a well or tray.

Sources of cells or tissues include human, all other animals, bacteria, fungi, viruses and plant. Tissues are complex targets and refer to a single cell type, a collection of cell types or an aggregate of cells generally of a particular kind. Tissue may be intact or modified. General classes of tissue in humans include but are not limited to epithelial tissue, connective tissue, nerve tissue and muscle tissue.

In another embodiment, the target is a cancer-related target that expresses CEA or that has CEA bound to itself or that has CEA located in its vicinity. The cancer-related target can be any target that a composition of the invention binds to as part of the diagnosis, detection or treatment of a cancer or cancer-associated condition in a subject, for example, a cancerous cell, tissue or organ, a molecule associated with a cancerous cell, tissue or organ, or a molecule, cell, tissue or organ that is associated with a cancerous cell, tissue or organ (e.g., a tumor-bound diagnostic or therapeutic molecule administered to a subject or to a biopsy taken from a subject, or a healthy tissue, such as vasculature, that is associated with cancerous tissue).

In a second aspect, the invention is drawn to a nucleic acid encoding a CAB molecule as set forth herein. The nucleic acid can be, for example, a DNA or an RNA. The present invention also provides a plasmid comprising a nucleic acid encoding a polypeptide comprising all or part of a CAB. The plasmid can be, for example, an expression plasmid that allows expression of the polypeptide in a host cell or organism, or *in vitro*. The expression vector can allow expression of the polypeptide in, for example, a bacterial cell. The bacterial cell can be, for example, an *E. coli* cell.

Because of the redundancy in the genetic code, typically a large number of DNA sequences encode any given amino acid sequence and are, in this sense, equivalent. As described below, it may be desirable to select one or another equivalent DNA sequences for use in an expression vector, based on the preferred codon usage of the host cell into which

the expression vector will be inserted. The present invention is intended to encompass all DNA sequences that encode the desired CAB.

An operable expression clone may be used and is constructed by placing the coding sequence in operable linkage with a suitable control sequence in an expression vector. The vector can be designed to replicate autonomously in the host cell or to integrate into the chromosomal DNA of the host cell. The resulting clone is used to transform a suitable host, and the transformed host is cultured under conditions suitable for expression of the coding sequence. The expressed CAB is then isolated from the medium or from the cells, although recovery and purification of the CAB may not be necessary in some instances.

Construction of suitable clones containing the coding sequence and a suitable control sequence employ standard ligation and restriction techniques that are well understood in the art. In general, isolated plasmids, DNA sequences or synthesized oligonucleotides are cleaved, modified and religated in the form desired. Suitable restriction sites can, if not normally available, be added to the ends of the coding sequence so as to facilitate construction of an expression clone.

Site-specific DNA cleavage is performed by treating with a suitable restriction enzyme (or enzymes) under conditions that are generally understood in the art and specified by the manufacturers of commercially available restriction enzymes. *See, e.g.*, product catalogs from Amersham (Arlington Heights, IL), Roche Molecular Biochemicals (Indianapolis, IN), and New England Biolabs (Beverly, MA). Incubation times of about one to two hours at a temperature that is optimal for the particular enzyme are typical. After each incubation, protein is removed by extraction with phenol and chloroform; this extraction can be followed by ether extraction and recovery of the DNA from aqueous fractions by precipitation with ethanol. If desired, size separation of the cleaved fragments may be performed by polyacrylamide gel or agarose gel electrophoresis using standard techniques. *See, e.g.*, Maxam *et al.*, 1980, *Methods in Enzymology* 65:499-560.

Ligations can be performed, for example, in 15-30 μ l volumes under the following standard conditions and temperatures: 20 mM Tris-Cl, pH 7.5, 10 mM MgCl₂, 10 mM DTT, 33 μ g/ml BSA, 10-50 mM NaCl, and either 40 μ M ATP and 0.01-0.02 (Weiss) units T4 DNA ligase at 0°C (for ligation of fragments with complementary single-stranded ends) or 1mM ATP and 0.3-0.6 units T4 DNA ligase at 14°C (for "blunt end" ligation).

Intermolecular ligations of fragments with complementary ends are usually performed at 33-100 µg/ml total DNA concentrations (5-100 nM total ends concentration).

Intermolecular blunt end ligations (usually employing a 20-30 fold molar excess of linkers, optionally) are performed at 1 µM total ends concentration.

Correct ligations for plasmid construction can be confirmed using any suitable method known in the art. For example, correct ligations for plasmid construction can be confirmed by first transforming a suitable host, such as *E. coli* strain DG101 (ATCC 47043) or *E. coli* strain DG116 (ATCC 53606), with the ligation mixture. Successful transformants are selected by ampicillin, tetracycline or other antibiotic resistance or sensitivity or by using other markers, depending on the mode of plasmid construction, as is understood in the art. Plasmids from the transformants are then prepared according to the method of Clewell *et al.*, 1969, Proc. Natl. Acad. Sci. USA 62:1159, optionally following chloramphenicol amplification. See Clewell, 1972, J. Bacteriol. 110:667. Alternatively, plasmid DNA can be prepared using the "Base-Acid" extraction method at page 11 of the Bethesda Research Laboratories publication Focus 5 (2), and very pure plasmid DNA can be obtained by replacing steps 12 through 17 of the protocol with CsCl/ethidium bromide ultracentrifugation of the DNA. As another alternative, a commercially available plasmid DNA isolation kit, *e.g.*, HISPEED™, QIAFILTER™ and QIAGEN® plasmid DNA isolation kits (Qiagen, Valencia CA) can be employed following the protocols supplied by the vendor. The isolated DNA can be analyzed by, for example, restriction enzyme digestion and/or sequenced by the dideoxy method of Sanger *et al.*, 1977, Proc. Natl. Acad. Sci. USA 74:5463, as further described by Messing *et al.*, 1981, Nuc. Acids Res. 9:309, or by the method of Maxam *et al.*, 1980, Methods in Enzymology 65:499.

The control sequences, expression vectors and transformation methods are dependent on the type of host cell used to express the gene. Generally, prokaryotic, yeast, insect or mammalian cells are used as hosts. Prokaryotic hosts are in general the most efficient and convenient for the production of recombinant proteins and are therefore preferred for the expression of the protein.

The prokaryote most frequently used to express recombinant proteins is *E. coli*. However, microbial strains other than *E. coli* can also be used, such as bacilli, for example *Bacillus subtilis*, various species of *Pseudomonas* and *Salmonella*, and other bacterial

strains. In such prokaryotic systems, plasmid vectors that contain replication sites and control sequences derived from the host or a species compatible with the host are typically used.

For expression of constructions under control of most bacterial promoters, *E. coli* K12 strain MM294, obtained from the *E. coli* Genetic Stock Center under GCSC #6135, can be used as the host. For expression vectors with the P_LN_RBS or P_L T7_RBS control sequence, *E. coli* K12 strain MC1000 lambda lysogen, N7N53cI857 SusP₈₀, ATCC 39531, may be used. *E. coli* DG116, which was deposited with the ATCC (ATCC 53606) on April 7, 1987, and *E. coli* KB2, which was deposited with the ATCC (ATCC 53075) on March 29, 1985, are also useful host cells. For M13 phage recombinants, *E. coli* strains susceptible to phage infection, such as *E. coli* K12 strain DG98 (ATCC 39768), are employed. The DG98 strain was deposited with the ATCC on July 13, 1984.

For example, *E. coli* is typically transformed using derivatives of pBR322, described by Bolivar *et al.*, 1977, Gene 2:95. Plasmid pBR322 contains genes for ampicillin and tetracycline resistance. These drug resistance markers can be either retained or destroyed in constructing the desired vector and so help to detect the presence of a desired recombinant. Commonly used prokaryotic control sequences, i.e., a promoter for transcription initiation, optionally with an operator, along with a ribosome binding site sequence, include the β -lactamase (penicillinase) and lactose (lac) promoter systems, *see* Chang *et al.*, 1977, Nature 198:1056, the tryptophan (trp) promoter system, *see* Goeddel *et al.*, 1980, Nuc. Acids Res. 8:4057, and the lambda-derived P_L promoter, *see* Shimatake *et al.*, 1981, Nature 292:128, and gene N ribosome binding site (N_RBS). A portable control system cassette is set forth in U.S. Patent No. 4,711,845, issued December 8, 1987. This cassette comprises a P_L promoter operably linked to the N_RBS in turn positioned upstream of a third DNA sequence having at least one restriction site that permits cleavage within six base pairs 3' of the N_RBS sequence. Also useful is the phosphatase A (phoA) system described by Chang *et al.*, in European Patent Publication No. 196,864, published October 8, 1986. However, any available promoter system compatible with prokaryotes can be used to construct an expression vector of the invention.

In addition to bacteria, eukaryotic microbes, such as yeast, can also be used as recombinant host cells. Laboratory strains of *Saccharomyces cerevisiae*, Baker's yeast, are most often used, although a number of other strains are commonly available. While vectors employing the two micron origin of replication are common, *see* Broach, 1983, Meth. Enz. 101:307, other plasmid vectors suitable for yeast expression are known. *See, e.g.,* Stinchcomb *et al.*, 1979, Nature 282:39; Tschempe *et al.*, 1980, Gene 10:157; and Clarke *et al.*, 1983, Meth. Enz. 101:300. Control sequences for yeast vectors include promoters for the synthesis of glycolytic enzymes. *See* Hess *et al.*, 1968, J. Adv. Enzyme Reg. 7:149; Holland *et al.*, 1978, Biotechnology 17:4900; and Holland *et al.*, 1981, J. Biol. Chem. 256:1385. Additional promoters known in the art include the promoter for 3-phosphoglycerate kinase, *see* Hitzeman *et al.*, 1980, J. Biol. Chem. 255:2073, and those for other glycolytic enzymes, such as glyceraldehyde 3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase and glucokinase. Other promoters that have the additional advantage of transcription controlled by growth conditions are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism and enzymes responsible for maltose and galactose utilization.

Terminator sequences may also be used to enhance expression when placed at the 3' end of the coding sequence. Such terminators are found in the 3' untranslated region following the coding sequences in yeast-derived genes. Any vector containing a yeast-compatible promoter, origin of replication and other control sequences is suitable for use in constructing yeast expression vectors.

The coding sequence can also be expressed in eukaryotic host cell cultures derived from multicellular organisms. *See, e.g.,* Tissue Culture, Academic Press, Cruz and Patterson, editors (1973). Useful host cell lines include COS-7, COS-A2, CV-1, murine cells such as murine myelomas N51 and VERO, HeLa cells and Chinese hamster ovary (CHO) cells. Expression vectors for such cells ordinarily include promoters and control sequences compatible with mammalian cells such as, for example, the commonly used early and late promoters from Simian Virus 40 (SV 40), *see* Fiers *et al.*, 1978, Nature 273:113, or other viral promoters such as those derived from polyoma, adenovirus 2,

bovine papilloma virus (BPV) or avian sarcoma viruses, or immunoglobulin promoters and heat shock promoters.

Enhancer regions are also important in optimizing expression; these are, generally, sequences found upstream of the promoter region. Origins of replication may be obtained, if needed, from viral sources. However, integration into the chromosome is a common mechanism for DNA replication in eukaryotes.

Plant cells can also be used as hosts, and control sequences compatible with plant cells, such as the nopaline synthase promoter and polyadenylation signal sequences, *see* Depicker *et al.*, 1982, J. Mol. Appl. Gen. 1:561, are available. Expression systems employing insect cells utilizing the control systems provided by baculovirus vectors have also been described. *See* Miller *et al.*, in Genetic Engineering (1986), Setlow *et al.*, eds., Plenum Publishing, Vol. 8, pp. 277-97. Insect cell-based expression can be accomplished in *Spodoptera frugiperda*. These systems are also successful in producing recombinant enzymes.

Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described by Cohen, 1972, Proc. Natl. Acad. Sci. USA 69:2110, is used for prokaryotes or other cells that contain substantial cell wall barriers. Infection with *Agrobacterium tumefaciens*, *see* Shaw *et al.*, 1983, Gene 23:315, is used for certain plant cells. For mammalian cells, the calcium phosphate precipitation method of Graham *et al.*, 1978, Virology 52:546 is preferred. Transformations into yeast are carried out according to the method of Van Solingen *et al.*, 1977, J. Bact. 130:946, and Hsiao *et al.*, 1979, Proc. Natl. Acad. Sci. USA 76:3829.

It may be desirable to modify the sequence of a DNA encoding a polypeptide comprising all or part of a CAB of the invention to provide, for example, a sequence more compatible with the codon usage of the host cell without modifying the amino acid sequence of the encoded protein. Such modifications to the initial 5-6 codons may improve expression efficiency. DNA sequences which have been modified to improve expression efficiency, but which encode the same amino acid sequence, are considered to be equivalent and encompassed by the present invention.

A variety of site-specific primer-directed mutagenesis methods are available and well-known in the art. See, e.g., Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, 1989, second edition, chapter 15.51, "Oligonucleotide-mediated mutagenesis," which is incorporated herein by reference. The polymerase chain reaction (PCR) can be used to perform site-specific mutagenesis. In another technique now standard in the art, a synthetic oligonucleotide encoding the desired mutation is used as a primer to direct synthesis of a complementary nucleic acid sequence contained in a single-stranded vector, such as pBSM13+ derivatives, that serves as a template for construction of the extension product of the mutagenizing primer. The mutagenized DNA is transformed into a host bacterium, and cultures of the transformed bacteria are plated and identified. The identification of modified vectors may involve transfer of the DNA of selected transformants to a nitrocellulose filter or other membrane and the "lifts" hybridized with kinased synthetic mutagenic primer at a temperature that permits hybridization of an exact match to the modified sequence but prevents hybridization with the original unmutagenized strand. Transformants that contain DNA that hybridizes with the probe are then cultured (the sequence of the DNA is generally confirmed by sequence analysis) and serve as a reservoir of the modified DNA.

Once the polypeptide has been expressed in a recombinant host cell, purification of the polypeptide may be desired. A variety of purification procedures can be used.

In another embodiment, a nucleic acid encoding the CAB hybridizes to a nucleic acid complementary to a nucleic acid encoding any of the amino acid sequences disclosed herein under highly stringent conditions. The highly stringent conditions can be, for example, hybridization to filter-bound DNA in 0.5 M NaHPO₄, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65° C and washing in 0.1xSSC/0.1 % SDS at 68° C (Ausubel *et al.*, eds., 1989, *Current Protocols in Molecular Biology*, Vol. I, Green Publishing Associates, Inc., and John Wiley & Sons, Inc., New York, at p. 2.10.3). Other highly stringent conditions can be found in, for example, *Current Protocols in Molecular Biology*, at pages 2.10.1-16 and *Molecular Cloning: A Laboratory Manual*, 2d ed., Sambrook *et al.* (eds.), Cold Spring Harbor Laboratory Press, 1989, pages 9.47-57. In another embodiment, moderately stringent conditions are used. The moderately stringent conditions can be, for example, washing in 0.2xSSC/0.1% SDS at 42° C (Ausubel *et al.*, 1989, *supra*). Other

moderately stringent conditions can be found in, for example, *Current Protocols in Molecular Biology*, Vol. I, Ausubel *et al.* (eds.), Green Publishing Associates, Inc., and John Wiley & Sons, Inc., 1989, pages 2.10.1-16 and *Molecular Cloning: A Laboratory Manual*, 2d ed., Sambrook *et al.* (eds.), Cold Spring Harbor Laboratory Press, 1989, pages 9.47-57.

In a third aspect the present invention provides a method of treating a subject in need thereof comprising administering to a subject a CAB and a prodrug that is a substrate of the CAB. In another embodiment, the invention provides a method of treating a subject by administering to the subject a CAB, further comprising a BLA, and a prodrug that is converted by the BLA into an active drug. In another embodiment, the CAB is specifically CAB1.11 or CAB1.11i.

Melphalan derivatives are especially suitable as the prodrug for this embodiment of the invention. Examples of enzyme/prodrug/active drug combinations can be found in, *e.g.*, Senter *et al.*, United States patent 5,773,435, which is incorporated by reference herein, including any drawings. Other examples of suitable prodrugs for this embodiment are provided in, *e.g.*, Melton *et al.*, *Enzyme-Prodrug Strategies for Cancer Therapy*, Kluwer Academic/Plenum Publishers, New York (1999), Bagshawe *et al.*, *Current Opinion in Immunology* 11:579-83 (1999) and Kerr *et al.*, *Bioconjugate Chem.* 9:255-59 (1998). Wilman, "Prodrugs In Cancer Chemotherapy," *Biochemical Society Transactions*, 14, pp. 375-82 (615th Meeting, Belfast 1986) and V. J. Stella *et al.*, "Prodrugs: A Chemical Approach To Targeted Drug Delivery," *Directed Drug Delivery*, R. Borchardt *et al.* (ed), pp.247-67 (Humana Press 1985).

In one embodiment, the prodrug is a peptide. Examples of peptides as prodrugs can be found in Trouet *et al.*, *Proc Natl Acad Sci USA* 79:626 (1982), and Umemoto *et al.*, *Int J Cancer* 43:677 (1989). These and other reports show that peptides are sufficiently stable in blood. Another advantage of peptide-derived prodrugs is their amino acid sequences can be chosen to confer suitable pharmacological properties like half-life, tissue distribution and low toxicity to the active drugs. Most reports of peptide-derived prodrugs relied on relatively nonspecific activation of the prodrug by, for instance, lysosomal enzymes.

The prodrug can be one that is converted to an active drug in more than one step. For example, the prodrug can be converted to a precursor of an active drug by the CAB.

The precursor can be converted into the active drug by, for example, the catalytic activity of one or more additional CABs, the catalytic activities of one or more other enzymes administered to the subject, the catalytic activity of one or more enzymes naturally present in the subject or at the target site in the subject (e.g., a protease, a phosphatase, a kinase or a polymerase), by a drug that is administered to the subject or by a chemical process that is not enzymatically catalyzed (e.g., oxidation, hydrolysis, isomerization or epimerization).

Most studies involving prodrugs are generated after programs with existing drugs are found to be problematic. In particular anticancer drugs were generally characterized by a very low therapeutic index. By converting these drugs into prodrugs with reduced toxicity and then selectively activating them in the diseased tissue, the therapeutic index of the drug was significantly increased. See, e.g., Melton *et al.*, *Enzyme-prodrug strategies for cancer therapy* (1999), and Niculescu-Duvaz *et al.*, *Anticancer Drug Des* 14:517 (1999).

The literature describes many methods to alter the substrate specificity of enzymes by protein engineering or directed evolution. Thus one skilled in the art is able to evolve the specificity of an enzyme to accommodate even structures that would be poor substrates for naturally-occurring enzymes. Accordingly, prodrugs can be designed even though the drugs were otherwise not amenable to a prodrug strategy.

The prodrugs of the invention can include, but are not limited to, auristatins, camptothecins, phosphate-containing prodrugs, thiophosphate-containing prodrugs, sulfate-containing prodrugs, peptide-containing prodrugs, D-amino acid-modified prodrugs, glycosylated prodrugs, β -lactam-containing prodrugs, optionally substituted phenoxyacetamide-containing prodrugs or optionally substituted phenylacetamide-containing prodrugs, 5-fluorocytosine and other 5-fluorouridine prodrugs which can be converted by the enzyme of the conjugate into the more active cytotoxic free drug.

Examples of cytotoxic drugs that can be derivatized into a prodrug form for use in this invention include, but are not limited to, etoposide, temposide, adriamycin, daunomycin, carminomycin, aminopterin, dactinomycin, mitomycins, cis-platinum and cis-platinum analogues, bleomycins, esperamicins (see U.S. Pat. No. 4,675,187), 5-fluorouracil, melphalan, other related nitrogen mustards and derivatives thereof. (See, e.g., U.S. Pat. No. 4,975,278).

In one embodiment of the invention, the CAB comprises an alkaline phosphatase (AP) that converts a 4'-phosphate derivative of the epipodophyl-lotoxin glucosides into an active anti-cancer drug. Such derivatives include etoposide-4'-phosphate, etoposide-4'-thiophosphate and teniposide-4'-phosphate. Other embodiments of the invention may include phosphate derivatives of these glucosides wherein the phosphate moiety is placed at other hydroxyl groups on the glucosides. According to another embodiment, however, the phosphate derivative used as a pro-drug in this invention is etoposide-4'-phosphate or etoposide-4'-thiophosphate. The targeted AP removes the phosphate group from the prodrug, releasing an active antitumor agent. The mitomycin phosphate prodrug of this embodiment may be an N⁷-C₁₋₈ alkyl phosphate derivative of mitomycin C or porfiromycin or pharmaceutically acceptable salts thereof. N⁷ refers to the nitrogen atom attached to the 7-position of the mitosane nucleus of the parent drug. According to another embodiment, the derivative used is 7-(2'-aminoethylphosphate)mitomycin ("MOP"). Alternatively, the MOP compound may be termed, 9-methoxy-7-[[[(phos-phonoxy)ethyl]amino]mitosane disodium salt. Other embodiments of the invention may include the use of N⁷-alkyl mitomycin phosphorothioates as prodrugs.

In still another embodiment of the invention, the CAB comprises a penicillin amidase enzyme that converts a novel adriamycin prodrug into the active antitumor drug adriamycin. In another embodiment, the penicillin amidase is a penicillin V amidase ("PVA") isolated from *Fusarium oxysporum* that hydrolyzes phenoxyacetyl amide bonds. The prodrug utilized can be N-(p-hydroxyphenoxyacetyl)adriamycin ("APO"), which is hydrolyzed by the amidase to release the potent antitumor agent or adriamycin.

The present invention also comprises, for example, the use of the adriamycin prodrug, N-(p-hydroxyphenoxyacetyl)adriamycin and other related adriamycin prodrugs that can be derivatized in substantially the same manner. For example, use of the prodrug N-(phenoxyacetyl) adriamycin is also within the scope of the invention. In addition, it is to be understood that the adriamycin prodrugs of this invention include other N-hydroxyphenoxyacetyl derivatives of adriamycin, e.g., substituted at different positions of the phenyl ring, as well as N-phenoxyacetyl derivatives containing substituents on the phenyl ring other than the hydroxyl group described herein.

Furthermore, the present embodiment encompasses the use of other amidases, such as penicillin G amidase, as part of the CAB as well as other prodrugs correspondingly derivatized such that the particular amidase can hydrolyze that prodrug to an active antitumor form. For example, when the CAB further comprises penicillin G amidase, the prodrug should contain a phenylacetylamine group (as opposed to the phenoxyacetylamine group of APO) because penicillin G amidases hydrolyze this type of amide bond (*see, e.g., A. L. Margolin et al., Biochim. Biophys. Acta.* 616, pp. 283-89 (1980)). Thus, other prodrugs of the invention include N-(p-hydroxyphenylacetyl) adriamycin, N-(phenylacetyl) adriamycin and other optionally substituted N-phenylacetyl derivatives of adriamycin.

It should also be understood that the present invention includes any prodrug derived by reacting the amine group of the parent drug with the carboxyl group of phenoxyacetic acid, phenylacetic acid or other related acids. Thus, prodrugs of anthracyclines other than adriamycin that are capable of being derivatized and acting in substantially the same manner as the adriamycin prodrugs described herein falls within the scope of this invention. For example, other prodrugs that can be produced and used in accordance with this invention include hydroxyphenoxyacetylamine derivatives, hydroxyphenylacetylamine derivatives, phenoxyacetylamine derivatives and phenylacetylamine derivatives of anthracyclines such as daunomycin and carminomycin. Other amine-containing drugs such as melphalan, mitomycin, aminopterin, bleomycin and dactinomycin can also be modified described herein to yield prodrugs of the invention.

Another embodiment of the invention involves a CAB form of the enzyme cytosine deaminase ("CD"). The deaminase enzyme catalyzes the conversion of 5-fluorocytosine ("5-FC"), a compound lacking in antineoplastic activity, to the potent antitumor drug, 5-fluorouracil ("5-FU").

Another embodiment of the method of this invention provides a method of combination chemotherapy using several prodrugs and a single CAB. According to this embodiment, a number of prodrugs are used that are all substrates for the same CAB. Thus, a particular CAB converts a number of prodrugs into cytotoxic form, resulting in increased antitumor activity at the tumor site.

There is often a requirement for extending the blood circulation half-lives of pharmaceutical peptides, proteins, or small molecules. Typically short half-lives—lasting

minutes to hours—require not only frequent, but also high doses for therapeutic effect—often so high that initial peak doses cause side effects. Extending the half-life of such therapeutics permits lower, less frequent, and therefore potentially safer doses, which are cheaper to produce. Previously researchers have increased protein half-life by fusing them covalently to PEG, *see* U.S. Patent 5,711,944, human blood serum albumin, *see* U.S. Patent 5,766,883, or Fc fragments, *see* WO 00/24782. In addition, nonspecific targeting of drugs to human serum albumin has been accomplished by chemical coupling drugs *in vivo*. *See* U.S. Patent 5,843,440. Furthermore, in the case of cancer drugs it has been proposed that high molecular weight drugs may localize in tumors due to enhanced permeability and retention. Therefore, improvement in the therapeutic index of a drug can be obtained by linking the drug to a protein or other high molecular weight polymer.

In another embodiment the present invention provides a method of treating a condition in a subject in need thereof, comprising administering to the subject a CAB with β -lactamase activity and a prodrug. In one embodiment, the subject in need thereof is a cancer patient. In another embodiment, the CAB is targeted to a CEA expressing cell, tissue, tumor or organ. In another embodiment, the prodrug is converted by the CAB into an active drug. In another embodiment, the active drug is an alkylating agent. In another embodiment, the prodrug is an anticancer nitrogen mustard prodrug. In another embodiment, the active drug is melphalan. In another embodiment, the prodrug is C-Mel. In another embodiment, the prodrug is glutaryl-C-Mel or glutaryl-C-Mel-L-Phe-NH₂ (*see*, for example, Senter *et al*, United States patent 5,773,435, which is incorporated by reference herein, including any drawings and Kerr *et al.*, *Bioconjugate Chem.* 9:255-59 (1998)). In another embodiment, the prodrug is vinca-cephalosporin or doxorubicin cephalosporin. *See* Bagshawe *et al.*, *Current Opinion in Immunology*, 11:579-83 (1999). Other prodrug/enzyme combinations that can be used in the present invention include, but are not limited to, those found in U.S. Patent No. 4,975,278 and Melton *et al.*, *Enzyme-Prodrug Strategies for Cancer Therapy* Kluwer Academic/Plenum Publishers, New York (1999).

In a fourth aspect, the invention is drawn to a pharmaceutical composition comprising a CAB molecule. The CABs, nucleic acids encoding them and, in certain embodiments, prodrugs described herein can be incorporated into pharmaceutical

compositions suitable for administration. Such compositions typically comprise the active compound and a pharmaceutically acceptable carrier. As used herein the language "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

The invention includes methods for preparing pharmaceutical compositions for modulating the expression or activity of a CAB, prodrug or nucleic acid of interest. Such methods comprise formulating a pharmaceutically acceptable carrier with an agent that modulates expression or activity of an active compound of interest. Such compositions can further include additional active agents. Thus, the invention further includes methods for preparing a pharmaceutical composition by formulating a pharmaceutically acceptable carrier with an agent that modulates expression or activity of a CAB, prodrug or nucleic acid of interest and one or more additional active compounds.

A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, *e.g.*, intravenous, intradermal, subcutaneous, oral (*e.g.*, inhalation), transdermal (topical), transmucosal and rectal administration. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. For intravenous administration, suitable carriers include normal saline, Water for Injection, 5% dextrose or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like) and suitable mixtures thereof. The proper fluidity can be maintained by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, sodium chloride in the composition.

Sterile injectable solutions can be prepared by incorporating the active compound in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation is freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Oral compositions generally include an inert diluent or an edible carrier. They can be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches or capsules.

Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as

microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate or orange flavoring.

For administration by inhalation, the compounds are delivered in the form of an aerosol spray from a pressurized container or dispenser that contains a suitable propellant, *e.g.*, a gas such as carbon dioxide or a nebulizer.

Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels or creams as generally known in the art.

The compounds can also be prepared in the form of suppositories (*e.g.*, with conventional suppository bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery.

In one embodiment, the active compounds are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811.

In one embodiment, the formulation comprises sulfobutylether-7-beta-cyclodextrin and 2-hydroxypropyl- β -cyclodextrin, as disclosed, for example, in United States patent

number 6,216,375 and United States patent number 6,537,988, each of which are incorporated by reference, herein, including any drawings.

It is especially advantageous to formulate oral or parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on the unique characteristics of the active compound and the particular therapeutic effect to be achieved and the limitations inherent in the art of compounding such an active compound for the treatment of individuals.

Typically, the amount of CAB to be delivered to a subject will depend on a number of factors, including, for example, the route of administration, the activity of the CAB, the degree to which it is specifically targeted to the desired cells, tissues or organs of the subject, the length of time required to clear the non-specifically bound CAB from the subject, the desired therapeutic effect, the body mass of the subject, the age of the subject, the general health of the subject, the sex of the subject, the diet of the subject, the subject's immune response to the CAB, other medications or treatments being administered to the subject, the severity of the disease and the previous or future anticipated course of treatment.

For applications in which a prodrug also is administered, other factors affecting the determination of a therapeutically effective dose will include, for example, the amount of prodrug administered, the activity of the prodrug and its corresponding active drug and the side effects or toxicities of the prodrug and the active drug.

Examples of ranges of mass of CAB/mass of subject include, for example, from about 0.001 to 30 mg/kg body weight, from about 0.01 to 25 mg/kg body weight, from about 0.1 to 20 mg/kg body weight, and from about 1 to 10 mg/kg, 2 to 9 mg/kg, 3 to 8 mg/kg, 4 to 7 mg/kg, or 5 to 6 mg/kg body weight.

In a particular example, a subject is treated with a CAB in the range of between about 0.1 to 20 mg/kg body weight, one time per week for between about 1 to 10 weeks, preferably between 2 to 8 weeks, preferably between about 3 to 7 weeks and preferably for

about 4, 5, or 6 weeks. It will also be appreciated that the effective dosage of CAB may increase or decrease over the course of a particular treatment, and that the treatment will continue, with or without modification, until a desired result is achieved or until the treatment is discontinued for another reason. Changes in dosage may result and become
5 apparent from the results of diagnostic assays as described herein.

It is understood that appropriate doses of prodrugs depend upon a number of factors within the ken of the ordinarily skilled physician, veterinarian or researcher. The dose(s) of the prodrug will depend, for example, on the same factors provided above as factors affecting the effective dose of the CAB. Exemplary doses include milligram or microgram
10 amounts of the prodrug per kilogram of subject or sample weight (*e.g.*, about 1 microgram per kilogram to about 500 milligrams per kilogram, about 100 micrograms per kilogram to about 5 milligrams per kilogram or about 1 microgram per kilogram to about 50 micrograms per kilogram). It is furthermore understood that appropriate doses of a prodrug depend upon the potency of the prodrug with respect to the desired therapeutic effect.

15 When one or more of these prodrugs is to be administered to an animal (*e.g.*, a human), a physician, veterinarian or researcher may, for example, prescribe a relatively low dose at first, subsequently increasing the dose until an appropriate response is obtained.

Preferably, the CAB is administered to the subject, then the prodrug is administered. More preferably, the time between the administration of the CAB and administration of the
20 prodrug is sufficient to allow the CAB to accumulate at its target site by binding to its target, and to allow unbound CAB to be cleared from the non-targeted portions of the subject's body. Most preferably, the ratio of target-bound CAB to unbound CAB in the subject's body will be at or near its maximum when the prodrug is administered. The time necessary after administration of the CAB to reach this point is called the clearing time.

25 The clearing time can be determined or approximated in an experimental system by, for example, administering a detectable CAB (*e.g.*, a radiolabeled or fluorescently labeled CAB) to a subject and simultaneously measuring the amount of enzyme at the target site and at a non-targeted control site at timed intervals. For some prodrugs, particularly those whose counterpart active drugs are highly toxic, it may be more important to ensure that the
30 levels of unbound CAB in the subject's system are below a certain threshold. This too can be determined experimentally, as described above.

In one embodiment, administration of the prodrug is systemic. In another embodiment, administration of the prodrug is at or near the target.

The pharmaceutical compositions can be included in a container, pack, dispenser or kit together with instructions for administration.

5

EXAMPLES

Example 1: Construction of CAB1.10

The amino acid sequence of the scFv portion of CAB1.10 molecule was derived from murine anti-CEA monoclonal antibody (MAb) T84.66 sequence (Neumaier et. al., 10 (1990) Cancer Research 50:2128-2134). The nucleotide sequence of the synthetic gene was designed based on E. coli codon usage plus a 30-aa linker connecting vL and vH domains with the following genetic configuration: vL-(GGGGS)₆-vH. A 968-bp DNA fragment containing the designed gene was synthesized by DNA2.0 (Menlo Park, CA) with flanking NcoI and EcoRV restriction sites and cloned into their pDriveCloningVector resulting in 15 plasmid pG00229.

Plasmid pNA31.1 is a stuffer vector with an inactive BLA gene that was used to clone the scFv portion from plasmid pG00229. This was derived from plasmid pME27.1 (see WIPO publication WO03105757A2, which is incorporated by reference, herein, including any drawings) upon digestion with PstI enzyme to remove the 461-bp region 20 containing a large part of the exigent antibody and a small part of BLA followed by self-ligation.

Upon digestion of plasmids pG00229 and pNA31.1 with NcoI and EcoRV enzymes, a 0.9-kb insert fragment and a 4.3-kb vector fragment, respectively, were gel purified. They were then ligated, followed by transformation into E. coli TOP10F' (Invitrogen, Carlsbad, 25 CA) competent cells and selection on agar plates containing Luria-Bertani medium and 5 ppm chloramphenicol (cmp) and 0.1 ppm cephotaxim (CTX). Out of hundreds of colonies, six clones were checked for proper size and orientation of the scFv fragment by isolating plasmid DNAs and digesting them with EcoRI and SacI enzymes. With both enzymes, expected restriction patterns were observed for all six clones tested. After testing four of 30 them for expression and binding, plasmid pHR03.1 was selected for further engineering and named as CAB1.10 molecule harboring plasmid.

Example 2: Combinatorial Consensus mutagenesis of CAB1.10

To improve the expression of CAB1.10 protein, a combinatorial consensus mutagenesis CCM approach as described before (see Attorney Docket Number 816P, which is incorporated by reference in its entirety, including any drawings) was pursued by targeting 35 amino acid residues in the frame work regions of vL and vH domains using plasmid pHR03.1 as a template. These 35 residues (14 positions in vH and 21 positions in vL) were identified as being significantly different (<10% abundance) compared to a typical human antibody sequence. Using a modified version of Multi-site Quikchange Mutagenesis (Stratagene, CA) protocol as described before (see Attorney Docket Number 816P, which is incorporated by reference herein, including any drawings), CCM libraries HR12 and HR14 with combined primer concentrations of 2 uM and 0.4 uM, respectively, were constructed employing 35 phosphorylated primers as shown in table 1. After mutagenesis and DpnI digestion, 2.5 ul out of 25 ul PCR reaction mix was transformed into E. coli TOP10F' cells followed by selection on agar plates containing Luria-Bertani medium and 5 ppm chloramphenicol (cmp) and 0.1 ppm cephalexin (CTX). 100 clones from library HR12 and 200 clones from library HR14 were initially screened for improved expression in 96-well microtiter plates as described below resulting in the selection of clone HR14.8. Sequencing of this clone revealed that it recruited A12S and R72G mutations in the vL region of the scFv fragment. Complete sequencing of the entire fusion gene of clone HR14.8 revealed no additional mutations elsewhere in the gene. This clone HR14.8 (encoded and was named) the CAB1.11 molecule.

Table. 1. Sequence of primers used for combinatorial consensus mutagenesis (CCM) of CAB1.10 protein. Primer name corresponds to the amino acid to be changed in the light (L) or heavy (H) chain, its position, and the intended mutation (mutated codon shown in upper case). So, for example, LD1Q corresponds to Asp (D) at position 1 of the light (L) chain to be changed to Gln (Q). The numbering starts with the first residue of either light or heavy chains. All primers were designed to the sense strand. The LA12S and LR68G primers incorporate A12S and R72G mutations in the CAB1.11 protein, respectively, and are shown in bold.

Combinatorial Consensus Mutagenesis (CCM) Primers		
Name	Sequence (5'-3')	Length
LD1Q	CggccatggccCAGatcgtctgacccagagcccg	36
LI2S	GccatggccgacTCTgtctgacccagagcccgcaag	38
LS7P	GtctgacccagCCGccggcaagcctggctgtttcc	36
LA9S	AcccagagcccgTCTagctggctgtttccctgggc	36
LA12S	CgggcaagcctgTCTgtttccctgggccagctgcc	36
LM21I	CcagcgtgccactATCtctgcagagcgggtgagtc	36
LP43A	GaaaccgggtcagGCGccaaaactgctgatctatcg	36
LV60D	GtccggcatcccgGACcgttctccggctactggctc	36
LT65S	GtacgtttctccggtTCTggetctcgtactgattttacc	39
LG66K	CgtttctccgtactAAAatctcgactgattttaccctg	39
LR68G	CgggtactggetctGGTactgattttaccctgattate	38
LD70T	CtggctctgactACCTttaccctgattatcgacccg	38
LF71A	GgctctgactgatGCGaccctgattatcgaccggtg	39
LI74T	ActgattttaccctgACCatcgaccgggtggaagcagac	39
LD76S	TtaccctgattatcTCTccgggtggaagcagacgatgttg	39
LP77G	AccctgattatcgacGGTgtggaagcagacgatgttgcc	39
LV83E	GtgggaagcagacgatGAAgccacctactattgccagcag	39
LT85D	GcagacgatgttgccGACTactattgccagcagaccaac	39
LE105T	CggtactaaactgACCATcaaaagcggtgggtgtgtctgg	39
LI106V	TactaaactggagGTTaaaggcggtgggtgtgtgtgtgg	39
LK106aL	TaaactggagatcCTGggcgggtgggtgtgtgtgtgtgg	39
Name	Sequence (5'-3')	Length
HE13K	GgtgcggagctcgttAAAccgggcgcttctgtgaaactg	39
HN28T	ActgcactggtttcACCatcaagacacctacatgcac	39
HI29F	GcatctggtttcaacTTCaaggacacctacatgcactgg	39
HK30S	TctggtttcaacattTCTgacacctacatgcactgggtg	39

	Combinatorial Consensus Mutagenesis (CCM) Primers	
HK38R	TacatgcactgggtgAGAcacgcccggaacagggtctg	39
HR40A	CactgggtgaacaaGCgccggaacagggtctggagtgg	39
HE42G	TgaaacaaccccgGGTcagggtctggagtggatcggtc	39
HK66R	CcaaaattccagggtAGAgcaaccatcactgctgatacc	39
HA67F	AaatccagggtaaaTTCaccatcactgctgatacctcc	39
HS75K	CtgcctgatacctccAAAaacactgcttacctgcagctgac	40
HT82aN	GcttacctgcagctgAACtcctgactagcgaagacacc	39
HP94R	TttattactgcgctAGAttcggctactatgtcagcgattac	41
HF95G	TattactgcgctccgGGTggctactatgtcagcgattac	39
HS108L	TggggtcagggcaccCTGgttaccgttttagcacaccg	39

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Example 3: Screening of the HR14 library

Library pHR14 was plated onto agar plates containing LB medium and 5 mg/l chloramphenicol and 0.1 mg/l cephotaxime (CTX, Sigma). Colonies from each library and parent colonies were transferred into 96 well plates containing 100ul LB + 5ppm cmp.

- 5 Plates were incubated at 30 C in a humidified box with shaking for 48-72 hrs. On the day of screening, 100ul of B-Per reagent (PIERCE) was added into each well and shaken at room temperature for 30 min.

- 10 Target protein CEA (Bioscience International) was immobilized in a 96 well polystyrene plate by adding 100 of 5ug/ml CEA in 50mM NaHCO₃ and the plate was incubated at 4C overnight. The plate was then washed with PBST (PBS+0.1%Tween 20) and blocked with 300ul/well of 1% Casein in PBS for 2 hours at room temp. On the day of screening, the plate was washed with PBST, subsequently, 80ul/well of PBSO (PBS+0.125% Octylglucopyranoside) was first added into the plate, followed by 20ul of diluted B-Per extracted cell culture of each well from expression plate. The plate was
- 15 incubated at room temperature with gentle shaking for 1 hour. After 1 hour, the plate was washed with PBST, 200ul of BLA substrate (nitrocefin in PBSO) was added into each well, the bound CAB molecule was measured by monitoring hydrolysis of nitrocefin at wavelength 490nm.

- 20 Seven variants of interest from the primary screening were streaked out on LA+5ppm cmp agar plate. Colonies from each variant were inoculated in 5ml of LB+5ppm cmp. The tubes were incubated at 25C for 70 hours. The culture was pelleted and resuspended in B-Per reagent. Target protein CEA was immobilized in 96 well polystyrene plate at 5ug/ml of 100ul/well, the plate was then blocked with 1% Casein. On the day of screening, 80ul/well of PBS buffer pH7.1 was added into target plate, and 20ul/well of 2-
- 25 fold serial diluted B-Per extract was added to the target plate. The plates were incubated at room temperature for 1 hour and were then washed with PBST. 200ul of BLA substrate nitrocefin in PBSO was added into each well, the bound CAB molecules was measured by monitoring hydrolysis of nitrocefin at wavelength 490nm. Assay results are shown in Figure 6. The best variant, HR14.8 was chosen for further refinement.

Example 4: Epitope removal of BLA

The i-mune assay was performed on the sequence for beta-lactamase as described (US Pat. Appln. Ser. No. 09/060,872, filed 4/15/98). Human population-based identification of CD4+ T cell peptide epitope determinants. (Journal of Immunological Methods, 281:95-108). Community donor peripheral blood cell samples were used. Four CD4+ T cell epitopes were identified. For each epitope peptide sequence, critical residue testing was performed. Critical residue testing included both an alanine scan of the peptide sequences, as well as specific amino acid modifications guided by functional and structural constraints. Peptide epitope sequences that reduced the level of proliferation to background levels were chosen and incorporated into a DNA construct of the beta-lactamase enzyme sequence. Modified enzyme protein variants were expressed and purified, then tested for their ability to induce cellular proliferation using human peripheral blood cells in vitro. The variant that induced the lowest level of cellular proliferation in vitro (which included BLA-epitope removing mutations at sites K21A and S324A) was selected for inclusion in CAB1.11i, as described below.

Example 5: Construction of CAB1.11i

Plasmid pHR19.2 was constructed from template HR14.8 (CAB1.11) by the Multi-site Quikchange Mutagenesis protocol (Stratagene, CA) to recruit the BLA-epitope-removing mutations K21A and S324A in the BLA protein.

Using phosphorylated primers HR016F and HR017F (HR016F: 5'-[Phosp]GATTACCCGCTGATGGCGGCCAGTCTGTTCCAG-3'; HR017F: 5'-[Phosp]CTACTGGCGGGTTTGGCGGTACGTGGCCTTTATTCCTG-3') for recruiting mutations K21A and S324A, respectively, a multi-site Quikchange mutagenesis (Stratagene, CA) reaction was performed followed by digestion with DpnI enzyme. 2.5 ul out of 2.5 ul PCR product was transformed into E. coli TOP10F' competent cells followed by selection of transformants on LA+Cm5+0.1 CTX plates. Plasmid DNAs from 16 clones were isolated and sequenced to confirm the recruitment of both mutations into the same plasmid. Only 2 (pHR19.2 and pHR19.15) clones were found to have both mutations in the same plasmid. Complete sequencing of the entire fusion gene of plasmid pHR19.2

revealed no additional mutations elsewhere in the gene. Finally, plasmid pHR19.2 was selected as the molecule that encodes the CAB1.11i molecule.

Example 6: Expression of CAB1.11i

E Coli. strain EB101.1 was obtained as a random isolate of strain NL106. Strain NL106 was transformed with a plasmid directing the production of an ADEPT construct and cultured in a 14 liter fermentor. Isolates from the fermentor were tested in shake flasks for production of lactamase activity, and one isolate NL106EB was chosen as host. Strain NL106 was subjected to serial shake flask cultures in defined medium and a faster growing strain, EB101.1, was isolated.

One glycerol vial containing strain EB101.1 carrying plasmid pHR19.2 was used to inoculate a flask containing 600 ml of MDM+1% Glucose media, the ingredients being shown in Table 2. The flask was incubated at 30C and 150 rpm in an incubator shaker. The growth was monitored by sampling flask and measuring Absorbance at A550. When the broth reached a reading of approximately A550=1, the content of the flask was transferred to a seed fermenter containing the same medium. When the broth inside the seed fermenter reached a cell density of A550=7-8, then 600 ml was transferred to the production fermenter containing production medium as shown in Table 2. The production fermenter was pH controlled and fed a 60% Glucose solution. The production fermenter was allowed to run for 32-40 h until the amount of CAB1.11i reached its maximum. Typical production profiles are shown in Figure 7, where lactamase activity is measured in mg/ml.

Table 2: MDM+1% Glucose Medium (Flask and Seed Tank medium)

Ingredient	Concentration in g/L
K2HPO4	13.6
KH2PO4	13.6
MgSO4*7H2O	2
Citric Acid Monohydrate	2
Ferric Ammonium Citrate	0.3
(NH4)2SO4	3.2

Ingredient	Concentration in g/L
Monosodium Glutamate	3.75 or L-Serine at 2.11 g/L
L-Tryptophan	0.40
Trace Metal Solution	1 ml
Glucose	10

Table 3: Production Medium

Ingredient	Concentration in g/L
Calcium chloride dihydrate	0.1818
Monosodium Glutamate	3.75 or L-Serine at 2.11 g/L
L-Tryptophan	0.40
Potassium phosphate monobasic	13.63
Citric Acid monohydrate	1.818
Magnesium sulfate heptahydrate	1.82
Ferric Ammonium citrate	0.303
Trace Metal Solution	0.909
Mazu DF204	0.78
Glucose	10
Sulfuric Acid	As required to adjust pH to 7.0
Ammonium Hydroxide	To control the pH at 7.0 during the fermentation

Example 7: Purification of CAB1.11i

Preparation of high purity samples of CAB1.11i was achieved using the process outlined in Figure 8. This process was highly efficient as the end product at each step can be input into the next step without the need for pH adjustment, buffer exchange or salt removal.

Step 1: B-PER Cell Wall Disruption and 60% Ammonium Sulfate Cut

Add 2.5ml B-PER Reagent (in Phosphate Buffer, Pierce Biotechnology Inc., product #78266) per gram of frozen E. coli cell paste. Benzonase Nuclease (Novagen,

product #70664-3) is also added at a dilution of 1:1000 during this step to hydrolyze DNA. Mixture is stirred vigorously for 60 minutes at Room temperature.

Remove cell debris by centrifugation at 4° C for 20 minutes and 12,000 rpm.

Discard pellet.

- 5 Add 390 grams solid ammonium sulfate (Sigma, product# A-2939) per 1 liter of supernatant to achieve 60% saturation at 25° C. Stir 40 minutes at room temperature. Recover precipitated protein by centrifugation at 4° C for 20 minutes and 12,000 rpm. Solubilize pellet into TEA Buffer (20mM triethanolamine/0.5M NaCl, pH 7). The crude protein solution should be centrifuged at 14,000 rpm and filtered through a 0.22 µm filter
10 prior to loading onto the PBA column.

Step 2: CAB1.11i Protein Capture via PBA Affinity Chromatography

- 15 A 30 ml PBA column (m-Aminophenylboronic acid immobilized onto agarose beads from Sigma, product # A-8530) was 'cleaned' with 150ml borate buffer (0.5M borate/0.5M NaCl, pH7), and equilibrated with 150ml TEA buffer prior to loading crude protein. After loading sample, the column was washed with 150ml TEA buffer (5 column volumes).

After loading the sample, the column is washed with 150ml TEA buffer.

CAB protein is eluted with 150 ml borate buffer and collected in 10 ml fractions.

- 20 Eluted fractions are assayed for β-lactamase using the nitrocefin plate assay, described, for example, in WO 0247717A2.

Step 3: Removal of CAB Degradation Products via Hydrophobic Charge Induction

- 25 Chromatography 5ml of CAB protein eluted from the PBA column loaded directly onto a 7ml MEP HyperCel column (produced by BioSeptra) equilibrated in phosphate buffered saline (PBS). After loading the sample, the column was washed with 10 column volumes of PBS. CAB protein was eluted from the resin using a 10 column volume gradient elution with 75mM sodium citrate buffer at pH 5.2.

- 30 Eluted fractions assayed for β-lactamase using the nitrocefin plate assay, as set forth above.

Step 4: Size Exclusion Chromatography for Obtaining Pure Monomer CAB1.11i

5ml of concentrated CAB protein was loaded onto a Superdex 75 preparative grade column (Amersham Biosciences, product# 17-1070-01) equilibrated with PBS. Proteins were separated with a flow rate of 2ml/min. of PBS and collected in 5ml fractions.

Step 5: Removal of Endotoxin via Detoxi-Gel

1-4ml of concentrated CAB protein was loaded onto a 10 ml Detoxi-Gel (immobilized polymyxin-B, Pierce, product# 20339) column equilibrated with PBS. The sample was left bound to the resin for 2.5 hours before eluting with PBS. Collect 20 1ml fractions.

Assay individual fractions for β -lactamase using the nitrocefin plate assay and for endotoxin using the BioWhittaker QCL-1000 Chromogenic Endpoint LAL assay. Calculated endotoxin units per mg of CAB protein. The maximum limit for in vivo murine studies is 5 units/mg and, for PBMC immunogenicity assays, 0.2 units/mg.

Figure 9 shows an SDS PAGE analysis of CAB1.11i protein of the protein that was purified by the above described procedure.

Example 8: PBMC assay of CAB1.11i

In order to test the potential immunogenicity of the CAB1.11i protein, the protein was tested in the PBMC proliferation assay. Community donor PBMC samples were purchased from the Stanford University Blood Center (Palo Alto, CA) or from BloodSource (Sacramento). Each sample was tested for common human bloodborne pathogens. PBMC were isolated from the buffy coat samples by differential centrifugation using Lymphocyte Separation Media (Gibco). PBMC were adjusted to 4×10^6 per ml in 5% heat-inactivated human AB serum-containing RPMI 1640. Cultures were seeded at 2 ml per well in a 24 well plate (Costar). Purified proteins were added at 20 ug/ml final concentration, and the bulk cultures were incubated at 37°C, 5% CO₂ for 5 days. Five days was selected after testing cultures at 4, 5, 6 and 7 days. The optimum responses were seen at 5 days for most proteins, with the exception of robust secondary responses to proteins such as tetanus toxoid that often peaked at day 4. On day 5 the bulk cultures were resuspended and 100 ul aliquots of each culture were replicately plated into a 96 well plate.

From 4 to 12 replicates were performed for each bulk culture. Tritiated thymidine was added at 0.25 uCi per well, and the replicates were cultured for 6 hours. Cultures were harvested to glass filtermats (Wallac) and the samples were counted in a scintillation counter (Wallac TriBeta). The CPM for each bulk culture were averaged. A control well with no added protein provided background CPM for each donor. A stimulation index for each test was calculated by dividing the experimental CPM by the control. An SI of 1.0 indicated that there was no proliferation above the background level. All purified protein samples were prepared in house. All proteins were tested for endotoxin using a commercially available kit (Pierce). All samples were adjusted to 1-2 mg/ml protein in PBS and were filter sterilized.

Thirty-six community donor samples were tested with the CAB1.11i protein. The average stimulation index was 1.06 ± 0.25 . This value is not different from background proliferation (SI = 1.0). None of the thirty-six donors mounted a proliferative response greater than 1.99, the cut-off value for a positive response. This is in contrast to previously collected data for the unmodified beta-lactamase protein, which showed an average stimulation index of 2.35 ± 3.50 , and a 27% response rate in 26 community donor samples. The proliferation results for the CAB1.11i are lower than the stimulation index results for the beta-lactamase ($p = 0.03$). No donors mounted a stimulation index of 1.99 or greater when tested with the CAB1.11i protein, as compared to the 27% responses to the beta-lactamase protein. Finally, the PBMC data for staphylokinase, a protein known to cause immune responses in community donors, was an average stimulation index was 3.68 ± 2.16 , with a 70% response rate. The data for CAB1.11i is highly different from the staphylokinase data. These results are interpreted to suggest that the CAB1.11i protein is comparatively non-immunogenic when tested in this human cell based, in vitro proliferation assay.

Example 9: Binding of CAB1.11i to CEA

Purified CEA (Bioscience International) was immobilized onto 96 well Costar High binding plates by incubation with a 5ug/ml solution in 50mM NaHCO₃ buffer at pH 9.6. A blocking step using casein to prevent non-specific binding was performed. Samples of purified CAB1.11i protein were pre-tested for their BLA enzymatic activity against

nitrocefin substrate to determine the specific activity. CAB1.11i was diluted in 10 mM PBS buffer pH 7.1 to 30,000 units/ml concentration. A 2 fold serial dilution was prepared in the same buffer and 100 μ l aliquots were added to the wells (8 samples: 3000, 1500, 750, 375, 187, 94, 47, 24 units). The protein was allowed to bind to the plates at ambient temperature for 1.5 h. The wells were extensively washed with PBS buffer containing Tween-20. The amount of CAB1.11i protein bound to the plates was determined by monitoring the amount of BLA remaining on the wells. The nitrocefin substrate (200 μ l/well of 0.1 mg/ml solution) was added to the wells and the product of the reaction was recorded by measuring the Absorbance at 490nm over a 20 min incubation period (ambient temperature). The V_{\max} was determined for each protein concentration, and a binding curve was generated by plotted protein bound versus protein added, to determine the K_d apparent for the material.

Binding curves are plotted in Figure 10A. CAB1.11i concentration was determined from measured BLA activity using a pre-determined conversion factor.

For apparent $t_{1/2}$ determinations, the CAB1.11i protein bound following the above-described procedure was sequentially allowed to wash off by incubation of the wells in PBS buffer pH 7.1 at ambient temperature. At prescribed times (0, 40, 80, 120 min) the buffer on the wells was removed and replaced with a nitrocefin substrate solution, and the BLA activity was determined as described above. The enzyme activity remaining bound (relative to time zero) was calculated, and the percent BLA activity bound (remaining) was plotted versus time to determine the 50% retention time.

The results can be seen in Figure 10B.

Example 10: CAB1.11i Circular Dichroism

Circular-dichroism (CD) spectra were collected on an Aviv 215 spectrophotometer equipped with a 5-position thermoelectric cell holder supplied by Aviv. Buffer conditions were phosphate buffered saline at pH7.4 and protein concentration was 1 μ M. Data was collected from 265 to 195 nm every 1 nm with a 1 nm bandwidth in a 0.1 cm path length cell at 25°C. Data was collected for 5 seconds at each wavelength and three replicate spectra were averaged. The CD signal was converted to mean residue ellipticity (MRE). The CD

Spectrum of CAB1.11i is indicative of a folded protein with both alpha helix and beta strand secondary structural components.

Example 11: Pharmacokinetics and Tissue Distribution of CAB1.11i in Xenograft

5 Mouse Model of Human Colorectal Cancer

Ncr athymic nude mice), 18-22g, approximately 6-8 weeks of age, were implanted subcutaneously with approximately 2 million tumor-derived LS174T human colorectal cancer cells. When tumors reached approximately $> 250 \text{ mm}^3$, 12 animals were administered a single IV bolus injection of CAB1.11i (1 mg/kg) via the tail vein and 3
10 animals were untreated to provide control tissues. Three animals were anaesthetized and sacrificed at 0, 6, 12, 24 and 48 hr. Liver, kidney and tumor were harvested from each animal, snap frozen in liquid nitrogen and stored at approximately -70°C until analysis. Blood was collected via cardiac puncture onto EDTA. Blood samples were centrifuged to separate plasma that was then stored at approximately -70°C until analysis.

15 Tissue samples were homogenized on ice in PBS with 15 $\mu\text{g/mL}$ aprotinin (2 mL buffer:gram tissue). Homogenate was mixed with B-PER (1:1) (from Pierce) and centrifuged. CAB1.11i concentrations in the tissue supernatant and plasma samples were determined by measuring BLA activity using a nitrocefin assay.

20 The results of this experiment indicated that CAB1.11i was rapidly eliminated from plasma, liver and kidney and localized to the TLS174T tumor (Figure 12). High tumor to blood ratio of CAB1.11i concentrations were sustained and achieved (Figure 13).

Example 12: Efficacy of CAB1.11i in a Xenograft Mouse Model of Human Colorectal Cancer

25 Ncr athymic nude mice, 18-22g, approximately 6-8 weeks of age, were implanted subcutaneously with approximately 2 million tumor-derived LS174T human colorectal cancer cells (TLS174T). LS174T cells were obtained from ATCC, passaged through mice and re-isolated to generate TL174T. When tumors reach approximately $> 250 \text{ mm}^3$, ten mice each were administered nothing or CAB1.11i (1 or 0.25 mg/kg followed by
30 administration of, Glutaryl-C-Mel, GCR-2141, shown for example, in United States Patent

5,773,435, as (150 mg/kg) 24 hours after CAB administration. All drugs were administered by IV bolus injections via the tail vein. Tumors were measured twice weekly.

The results of this study demonstrated that CAB1.11i at both 1 m/kg and 0.25 mg/kg doses in combination with the prodrug GCR-2141 at 150 mg/kg was active in a mouse model of human colorectal cancer (*see* Figure 14).

Having described the preferred embodiments of the present invention, it will appear to those ordinarily skilled in the art that various modifications may be made to the disclosed embodiments, and that such modifications are intended to be within the scope of the present invention.

One skilled in the art would readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The molecular complexes and the methods, procedures, treatments, molecules, specific compounds described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. It will be readily apparent to one skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

All patents and publications mentioned in the specification are indicative of the levels of those skilled in the art to which the invention pertains. All patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

The invention illustratively described herein suitably may be practiced in the absence of any element or elements, limitation or limitations that is not specifically disclosed herein. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the concepts herein disclosed may be resorted to by those

skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the appended claims.

The invention has been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form
5 part of the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

ABSTRACT

The present invention relates to CAB molecules, ADEPT constructs directed against CEA, and their use in therapy.

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Figure 1A

1 DIVLTQSPAS LAVSLGQRAT MSCRAGESVD IFGVGFLHWY QQKPGQPPKL
51 LIYRASNLES GIPVRFSGTG SRTDFTLIID PVEADDVATY YCQQTNEDPY
101 TFGGGTKLEI KGGGSGGGG SGGGSGGGG SGGGSGGGG SEVQLQSGA
151 ELVEPGASVK LSCTASGFNI KDTYMHVVKQ RPEQGLEWIG RIDPANGNSK
201 YVPKFQ GKAT ITADTSSNTA YLQLTSLTSE DTAVYYCAPF GYVSDYAMA
251 YWGQGTSTVTV SS

Figure 1B

1	GACATCGTCC	TGACCCAGAG	CCCGGCAAGC	CTGGCTGTTT	CCCTGGGCCA
51	GCGTGCCACT	ATGTCTTGCA	GAGCGGGTGA	GTCTGTTGAC	ATTTTCGGTG
101	TCGGTTTCT	GCACTGGTAC	CAACAGAAAC	CGGGTCAGCC	GCCAAAAC TG
151	CTGATCTATC	GTGCTTCTAA	CCTGGAGTCC	GGCATCCCGG	TACGTTTCTC
201	CGGTACTGGC	TCTCGTACTG	ATTTTACCCT	GATTATCGAC	CCGGTGGAAG
251	CAGACGATGT	TGCCACCTAC	TATTGCCAGC	AGACCAACGA	GGATCCGTAC
301	ACCTTCGGTG	GCGGTACTAA	ACTGGAGATC	AAAGGCGGTG	GTGTTCTGCG
351	TGGTGGTGGT	AGCGGCGGCG	GTGGTAGCGG	TGGCGGTGGC	AGCGGTGGTG
401	GTGGCTCTGG	TGGCGGTGGC	TCTGAAGTGC	AGCTGCAGCA	GTCCGGTGGG
451	GAGCTCGTTG	AACCGGGGCGC	TTCTGTGAAA	CTGTCTTGCA	CTGCATCTGG
501	TTTCAACATT	AAGGACACCT	ACATGCACTG	GGTGAAACAA	CGCCCGGAAC
551	AGGGTCTGGA	GTGGATCGGT	CGCATCGATC	CGGCTAACGG	TAACAGCAAA
601	TACGTGCCAA	AATTCCAGGG	TAAAGCAACC	ATCACTGCTG	ATACCTCCTC
651	TAACACTGCT	TACCTGCAGC	TGACTTCCCT	GA CTAGCGAA	GACACCGCGG
701	TTTATTACTG	CGCTCCGTTT	GGCTACTATG	TCAGCGATTA	CGCAATGGCC
751	TACTGGGGTC	AGGGCACCTC	TGTTACCGTT	TCTAGC	

Figure 1C

263 TPVSEKQL AEVVANTITP LMKAQSVPGM AVAVIYQGKP
301 HYYTFGKADI AANKPVTPQT LFELGSISKT FTGVLGGDAI ARGEISLDDA
351 VTRYWPQLTG KQWQGIRMLD LATYTAGGLP LQVPDEVTDN ASLLRFYQNW
401 QPQWKPGTTR LYANASIGLF GALAVKPSGM PYEQAMTTRV LKPLKLDHTW
451 INVPKAEAAH YAWGYRDGKA VRVSPGMLDA QAYGVKTNVQ DMANWVMANM
501 APENVADASL KQGIALAQSR YWRIGSMYQG LGWEMLNWPV EANTVVETSF
551 GNVALAPLPV AEVNPPAPPV KASVWHKTGS TGGFGSYVAF IPEKQIGIVM
602 LANTSYPNPA RVEAAYHILE ALQ

Figure 1D

```
1  ACACCGGTGT CAGAAAAACA GCTGGCGGAG GTGGTCGCGA ATACGATTAC
51 CCCGCTGATG AAAGCCCAGT CTGTTCCAGG CATGGCGGTG GCCGTTATTT
101 ATCAGGGA AAA ACCGCACTAT TACACATTG GCAAGGCCGA TATCGCGGCG
151 AATAAACCCG TTACGCCTCA GACCCTGTTT GAGCTGGGTT CTATAAGTAA
201 AACCTTCACC GCGTTTTTAG GTGGGATGC CATTGCTCGC GGTGAAATTT
251 CGCTGGACGA TCGGTGACC AGATACTGGC CACAGCTGAC GGGCAAGCAG
301 TGGCAGGGTA TTCGTATGCT GGATCTCGCC ACCTACACCG CTGGCGGCCT
351 GCGCTACAG GTACCGGATG AGGTCACGGA TAACGCCTCC CTGCTGCGCT
401 TTTATCAAAA CTGGCAGCGG CAGTGAAGC CTGGCACAAC GCGTCTTTAC
451 GCCAACGCCA GCATCGGTCT TTTTGGTGCG CTGGCGGTCA AACCTTCTGG
501 CATGCCCTAT GAGCAGGCCA TGACGACGCG GTCCTTAAG CCGCTCAAGC
551 TGGACCATA CTGGATTAAC GTGCCGAAAG CGGAAGAGGC GCATTACGCC
601 TGGGGCTATC GTACCGGTAA AGCGGTGCGC GTTTCGCCGG GTATGCTGGA
651 TGCACAAGCC TATGGCGTGA AAACCAACGT GCAGGATATG GCGAACTGGG
701 TCATGGCAAA CATGGCGCCG GAGAACGTTG CTGATGCCTC ACTTAAGCAG
751 GGCATCGCGC TGGCGCAGTC GCGCTACTGG CGTATCGGGT CAATGTATCA
801 GGGTCTGGGC TGGGAGATGC TCAACTGGCC CGTGGAGGCC AACACGGTGG
851 TCGAGACGAG TTTTGCTAAT GTAGCACTGG CGCCGTGGCC CGTGGCAGAA
901 GTGAATCCAC CGGCTCCCCC GGTCAAAGCG TCCTGGGTCC ATAAACGGG
951 CTCTACTGGC GGGTTTGCA GCTACGTGGC CTTTATTCTT GAAAAGCAGA
1001 TCGGTATTGT GATGCTCGCG AATACAAGCT ATCCGAACCC GGCACGCGTT
1051 GAGGCGGCAT ACCATATCCT CGAGGCGCTA CAG
```


Figure 1E

1 DIVLTQSPAS LAVSLGQRAT MSCRAGESVD IFGVGFLHWY QQKPGQPPKL
51 LIYRASNLGS GIPVRFSGTG SRTDFTLIID PVEADDVATY YCQQTNEPDY
101 TFGGGTKLEI KGGGSGGGG SGGGSGGGG SGGGSGGGG SEVQLQQSGA
151 ELVEPGASVK LSCTASGFNI KDTYMHVWKQ RPEQGLEWIG RIDPANGNSK
201 YVPKFQ GKAT ITADTSSNTA YLQLTSLTSE DTAVYYCAPF GYYVSDYAMA
251 YWQGQTSVTV SSTPVSEKQL AEVVANTITP LMKAQSVPGM AVAVIYQGKP
301 HYYTFGKADI AANKPVTPTQ LFELGSISK TFGVLGGDAI ARGEISLDDA
351 VTRYWPQLTG KQWQGIRMLD LATYTAGGLP LQVPDEVDN ASLLRFYQNW
401 QPQWKPGTTR LYANASIGLF GALAVKPSGM PYEQAMTRV LKPLKLDHTW
451 INVPKAEAAH YAWGYRDGKA VRVSPGMLDA QAYGVKTNVQ DMANWVMANM
501 APENVADASL KQGIALAQSR YWRIGSMYQG LGWEMLNWPV EANTVVETSF
551 GNVALAPLPV AEVNPPAPPV KASWVHKTGS TGGFGSYVAF IPEKQIGIVM
601 LANTSYFNPA RVEAAYHILE ALQ

Figure 1F

1 GACATCGTCC TGACCCAGAG CCCGGCAAGC CTGGCTGTTT
CCCTGGGCCA
51 GCGTGCCACT ATGTCCTGCA GAGCGGGTGA GTCTGTTGAC
ATTTTCGGTG
101 TCGGTTTTCT GCACTGGTAC CAACAGAAAC CGGGTCAGCC
GCCAAAACTG
151 CTGATCTATC GTGCTTCTAA CCTGGAGTCC GGCATCCCGG
TACGTTTCTC
201 CGGTACTGGC TCTCGTACTG ATTTTACCCT GATTATCGAC
CCGGTGAAG
251 CAGACGATGT TGCCACCTAC TATTGCCAGC AGACCAACGA
GGATCCGTAC
301 ACCTTCGGTG GCGGTACTAA ACTGGAGATC AAAGCGGGTG
GTGGTTCTGG
351 TGGTGGTGGT AGCGGCGGCG GTGGTAGCGG TGGCGGTGGC
AGCGGTGGTG
401 GTGGCTCTGG TGGCGGTGGC TCTGAAGTGC AGCTGCAGCA
GTCCGGTGCG
451 GAGTCTCGTTG AACCGGGCGC TTCTGTGAAA CTGTCTTGCA
CTGCATCTGG
501 TTTCAACATT AAGGACACCT ACATGCACTG GGTGAAACAA
CGCCCGGAAC
551 AGGGTCTGGA GTGGATCGGT CGCATCGATC CGGCTAACGG
TAACAGCAAA
601 TACGTGCCAA AATTCCAGGG TAAAGCAACC ATCACTGCTG
ATACCTCCTC
651 TAACACTGCT TACCTGCAGC TGA CTTCCT GACTAGCGAA
GACACCGCGG
701 TTTATTACTG CGCTCCGTTC GGCTACTATG TCAGCGATTA
CGCAATGGCC
751 TACTGGGGTC AGGGCACCTC TGTTACCGTT TCTAGCACAC
CGGTGTCAGA
801 AAAACAGCTG GCGGAGGTGG TCGCGAATAC GATTACCCCG
CTGATGAAAG
851 CCCAGTCTGT TCCAGGCATG GCGGTGGCCG TTATTTATCA
GGGAAAACCG
901 CACTATTACA CATTTGGCAA GGCCGATATC GCGGCGAATA
AACCCGTTAC
951 GCCTCAGACC CTGTTTCGAGC TGGGTTCTAT AAGTAAACCC
TTCACCGGCG
1001 TTTTAGGTGG GGATGCCATT GCTCGCGGTG AAATTTCTGT
GGACGATGCG
1051 GTGACCAGAT ACTGGCCACA GCTGACGGGC AAGCAGTGGC
AGGGTATTCTG

1101 TATGCTGGAT CTCGCCACCT ACACCGCTGG CGGCCTGCCG
CTACAGGTAC
1151 CGGATGAGGT CACGGATAAC GCCTCCCTGC TGCGCTTTTA
TCAAAACTGG
1201 CAGCCGCACT GGAAGCCTGG CACAACGCGT CTTTACGCCA
ACGCCAGCAT
1251 CGGTCTTTTT GGTGCGCTGG CGGTCAAACC TTCTGGCATG
CCCTATGAGC
1301 AGGCCATGAC GACGCGGGTC CTTAAGCCGC TCAAGCTGGA
CCATACCTGG
1351 ATTAACGTGC CGAAAGCGGA AGAGGCGCAT TACGCTGGG
GCTATCGTGA
1401 CGGTAAAGCG GTGCGCGTTT CGCCGGGTAT GCTGGATGCA
CAAGCCTATG
1451 GCGTGAAAAC CAACGTGCAG GATATGGCGA ACTGGGTCAT
GGCAAACATG
1501 GCGCCGGAGA ACGTTGCTGA TGCCTCACTT AAGCAGGGCA
TCGCGCTGGC
1551 GCAGTCGCGC TACTGGCGTA TCGGGTCAAT GTATCAGGGT
CTGGGCTGGG
1601 AGATGCTCAA CTGGCCCGTG GAGGCCAACA CGGTGGTCGA
GACGAGTTTT
1651 GGTAATGTAG CACTGGCGCC GTTGCCCGTG GCAGAAGTGA
ATCCACCGGC
1701 TCCCCCGGTC AAAGCGTCTT GGGTCCATAA AACGGGCTCT
ACTGGCGGGT
1751 TTGGCAGCTA CGTGGCCTTT ATTCCTGAAA AGCAGATCGG
TATTGTGATG
1801 CTCGCGAATA CAAGCTATCC GAACCCGGCA CGCGTTGAGG
CGGCATACCA
1851 TATCCTCGAG GCGCTACAG

Figure 2A

DIVLTQSPAS LSVSLGQRAT MSCRAGESVD IFGVGFLHWY QQKPGQPPKL

51 LIYRASNLES GIPVRFSGTG SGTDFTLIID PVEADDVATY YCQQTNEDPY

101 TFGGGTKLEI KGGGGSGGGG SGGGGSGGGG SGGGGSGGGG SEVQLQQSGA

151 ELVEPGASVK LSCTASGFNI KDTYMHVKQ RPEQGLEWIG RIDPANGNSK

201 YVPKFQ GKAT ITADTSSNTA YLQLTSLTSE DTAVYYCAPF GYYVSDYAMA

251 YWGQGTSTVTV SS

Figure 2B

```
1   GACATCGTCC TGACCCAGAG CCCGGCAAGC CTGTCTGTTT CCCTGGGCCA
51  GCGTGCCACT ATGTCTTGCA GAGCGGGTGA GTCTGTTGAC ATTTTCGGTG
101 TCGGTTTTCT GCACTGGTAC CAACAGAAAC CGGGTCAGCC GCCAAAAC TG
151 CTGATCTATC GTGCTTCTAA CCTGGAGTCC GGCATCCCGG TACGTTTCTC
201 CGGTACTGGC TCTGGTACTG ATTTTACCCT GATTATCGAC CCGGTGGAAG
251 CAGACGATGT TGCCACCTAC TATTGCCAGC AGACCAACGA GGATCCGTAC
301 ACCTTCGGTG GCGGTACTAA ACTGGAGATC AAAGCGGGTG GTGGTTCCTG
351 TGGTGGTGGT AGCGGTGGCG GTGGTAGCGG TGGCGGTGGC AGCGGTGGTG
401 GTGGCTCTGG TGGCGGTGGC TCTGAAGTGC AGCTGCAGCA GTCCGTGGCG
451 GAGCTCGTTG AACCGGGCGC TTCTGTGAAA CTGTCTTGCA CTGCATCTGG
501 TTTCAACATT AAGGACACCT ACATGCACTG GGTGAAACAA CGCCCGGAAC
551 AGGGTCTGGA GTGGATCGGT CGCATCGATC CGGCTAACGG TAACAGCAAA
601 TACGTGCCAA AATTCCAGGG TAAAGCAACC ATCACTGCTG ATACCTCCTC
651 TAACACTGCT TACCTGCAGC TGACTTCCTT GACTAGCGAA GACACCGCGG
701 TTTATTACTG CGCTCCGTTT GGCTACTATG TCAGCGATTA CGCAATGGCC
751 TACTGGGGTC AGGGCACCTC TGTTACCGTT TCTAGC
```

Figure 3

262 TPVSEKQL AEVVANTITP LMAAQSVPGM AVAVIYQGKP
301 HYYTFGKADI AANKPVTPTQ LFELGSISKI FTGVLGGDAI ARGEISLDDA
351 VTRYWPQLTG KQWQGIRMLD LATYTAGGLP LQVPDEVTDN ASLLRFYQNW
401 QPQWKPGTTR LYANASIGLF GALAVKPSGM PYEQAMTTRV LKPLKLDHTW
451 INVPKAEAAH YAWGYRDGKA VRVSPGMLDA QAYGVKTNVQ DMANWVMANM
501 APENVADASL KQGIALAQSR YWRIGSMYQG LGWEMLNWPV EANTVVETSF
551 GNVALAPLPV AEVNPPAPPV KASWVHKTGS TGGFGAYVAF IPEKQIGIVM
601 LANTSYNPNA RVEAAYHILE ALQ

Figure 4A

1 DIVLTQSPAS LSVSLGQRAT MSCRAGESVD IFGVGFLHWY QQKPGQPPKL
51 LIYRASNLES GIPVRFSGTG SGTDFTLIID PVEADDVATY YCQQTNEDPY
101 TFGGGTKLEI KGGGSGGGG SGGGSGGGG SGGGSGGGG SEVQLQQSGA
151 ELVEPGASVK LSTASGFNI KDTYMHVVKQ RPEQGLEWIG RIDPANGNSK
201 YVPKFQ GKAT ITADTSSNTA YLQLTSLTSE DTAVYYCAPF GYYVSDYAMA
251 YWQGQTSVTV SSTPVSEKQL AEVVANTITP LMKAQSVPGM AVAVIYQGKP
301 HYYTFGKADI AANKPVTPQT LFELGSISKT FTGVLGGDAI ARGEISLDDA
351 VTRYWPQLTG KQQGIRMLD LATYTAGGLP LQVPDEVDN ASLLRFYQNW
401 QPQWKPGTTR LYANASIGLF GALAVKPSGM PYEQAMTTRV LKPLKLDHTW
451 INVPKAEEAH YAWGYRDGKA VRVSPGMLDA QAYGVKTNVQ DMANWVMANM
501 APENVADASL KQGIALAQSR YWRIGSMYQG LGWEMLNWPV EANTVVETSF
551 GNVALAPLPV AEVNPPAPFV KASWVHKTGS TGGFGSYVAF IPEKQIGIVM
601 LANTSYPNPA RVEAAYHILE ALQ

Figure 4B

```
1.   GACATCGTCC TGACCCAGAG CCCGGCAAGC CTGTCTGTTT CCCTGGGCCA
51  GCGTGCCACT ATGTCTCTGA GAGCGGGTGA GTCTGTTGAC ATTTTCGGTG
101 TCGGTTTTCT GCACCTGGTAC CAACAGAAAC CGGGTCAGCC GCCAAACTG
151 CTGATCTATC GTGCTTCTAA CCTGGAGTCC GGCATCCCGG TACGTTTCTC
201 CGGTACTGGC TCTGGTACTG ATTTTACCCT GATTATCGAC CCGGTGGAAG
251 CAGACGATGT TGCCACCTAC TATTGCCAGC AGACCAACGA GGATCCGTAC
301 ACCTTCGGTG GCGGTACTAA ATGGAGATC AAAGGCGGTG GTGGTTCGTG
351 TGGTGGTGGT AGCGGTGGCG GTGGTAGCGG TGGCGGTGGC AGCGGTGGTG
401 GTGGCTCTGG TGGCGGTGGC TCTGAAGTGC AGCTGCAGCA GTCCGGTGGC
451 GAGCTCGTTG AACCGGGCGC TTCTGTGAAA CTGTCTTGCA CTGCATCTGG
501 TTTCAACATT AAGGACACCT ACATGCACGT GGTGAAACAA CGCCCGGAAC
551 AGGGTCTGGA GTGGATCGGT OGCATCGATC CGGCTAACGG TAACAGCAAA
601 TACGTGCCAA AATTCCAGGG TAAAGCAACC ATCACTGCTG ATACCTCTCT
651 TAACACTGCT TACCTGCAGC TGACTTCCCT GACTAGCGAA GACACCGCGG
701 TTTATTACTG CGCTCCGTTT GGCTACTATG TCAGCGGATTA CGCAATGGCC
751 TACTGGGGTC AGGGCACCTC TGTTACCGTT TCTAGCACAC CGGTGTCAGA
801 AAAACAGCTG GCGGAGGTGG TCGCGAATAC GATTACCCCG CTGATGAAAG
851 CCCAGTCTGT TCCAGGCATG GCGGTGGCGC TTATTATCA GGGAAACCGG
901 CACTATTACA CATTTGGCAA GGCCGATATC GCGCGAATA AACCCGTAC
951 GCCTCAGACC CTGTTTCGAGC TGGGTTCCTAT AAGTAAACCC TTCACCGCGG
1001 TTTTAGGTGG GGATGCCATT GCTCGCGGTG AAATTTCCGT GGACGATGCG
1051 GTGACCAGAT ACTGGCCACA GCTGACGGGC AAGCAGTTGG AGGCTATTTC
1101 TATGCTGGAT CTGCGCCACT ACACCGCTGG CGGCCTGCCG CTACAGGTAC
1151 CGGATGAGGT CACGGATAAC GCCTCCCTGC TGCCTTTTAA TCAAACTGG
1201 CAGCCGAGT GGAAGCCTGG CACAACGCGT CTTTACGCCA AGCCGAGCAT
1251 CGGTCTTTT GGTGCGCTGG CGGTCAAACC TTCTGGCATG CCTATGAGC
1301 AGGCCATGAC GACGCGGGTC CTTAAGCGGC TCAAGCTGGA CCATACCTGG
1351 ATTAACGTGC CGAAAGCGGA AGAGGCGCAT TACGCCTGGG GCTATCGTGA
1401 CGGTAAAGCG GTGCGCGTTT CGCCGGGTAT GCTGGATGCA CAAGCCTATG
1451 CGGTGAAAC CAACTGTCAG GATATGGCGA ACTGGGTGAT GGCAAAACATG
1501 GCGCCGGAGA ACGTTGCTGA TGCCTCACTT AAGCAGGGCA TCGCGCTGGC
1551 GCAGTCGCGC TACTGGCGTA TCGGTCAAAT GTATCAGGGT CTGGCTGGG
1601 AGATGCTCAA CTGGCCCGTG GAGGCCAACA CGGTGGTCTGA GACGAGTTTT
1651 GGTATGTAG CACTGGCGCC GTTGGCCGTG GCGAAGTGA ATCCACCGGC
1701 TCCCCCGGTC AAAGCGTCCT GGGTCCATAA AACGGGCTCT ACTGGCGGGT
1751 TTGGCAGCTA CGTGGCCTTT ATTCCTGAAA AGCAGATCGT TATTGTGATG
1801 CTCGCGAATA CAAGTATACC GAACCCGCGA CGCGTTGAGG CGGCATACCA
1851 TATCTCTGAG GCGCTACAG
```


Figure 4C

1 DIVLTQSPAS LSVSLGORAT MSCRAGESVD IFGVGFLHWY QQKPGPPKL
51 LIYRASNLES GIPVRFSGTG SGTDFTLIID PVEADDVATY YCQQTNEBPY
101 TFGGGTKLEI KGGGGSGGGG SGGGGSGGGG SGGGGSGGGG SEVQLQQSGA
151 ELVEPGASVK LSCTASGFNI KDTYMHVVKQ RPEQGLEWIG RIDPANGNSK
201 YVPKFQ GKAT ITADTSSNTA YLQLTSLTSE DTA VYYCAPF GYYVSDYAMA
251 YWGQGTSTTV SSTPVSEKQL AEVVANTITP LMAAQSVPGM AVAVIYQGKP
301 HYYTFGKADI AANKPVTPTQ LFELGSISKT FTGVLGGDAI ARGEISLDDA
351 VTRYWPQLTG KQWQGIRMLD LATYTAGGLP LQVPDEVTDN ASLLRFYQNW
401 QPQWKPGTTR LYANASIGLF GALAVKPSGM PYEQAMTRV LKPLKLDHTW
451 INVPKAEAAH YAWGYRDGKA VRVSPGMLDA QAYGVKTNVQ DMANWVMANM
501 APENVADASL KQGIALAQSR YWRIGSMYQG LGWEMLNWPV EANTVVETSF
551 GNVALAPLPV AEVNPPAPPV KASWVHKTGS TGGFGA YVAF IPEKQIGIVM
601 LANTSYPNPA RVEAAYHILE ALQ

Figure 4D

1	GACATCGTCC	TGACCCAGAG	CCCGGCAAGC	CTGTCTGTTT	CCCTGGGCCA
51	CGGTGCCACT	ATGTCTGCA	GAGCGGTGA	GTCTGTTGAC	ATTTTCGGTG
101	TCGGTTTCTT	GCACCTGGTAC	CAACAGAAAC	CGGGTCAGCC	GCCAAAACCTG
151	CTGATCTATC	GTGCTTCTAA	CCTGGAGTCC	GGCATCCCGG	TACGTTTCTC
201	CGGTACTGGC	TCTGGTACTG	ATTTTACCCT	GATTATCGAC	CCGGTGGGAG
251	CAGACGATGT	TGCCACCTAC	TATTGCCAGC	AGACCAACGA	GGATCCGTAC
301	ACCTTCGGTG	GCGGTACTAA	ACTGGAGATC	AAAGGCGGTG	GTGGTTCTGG
351	TGGTGGTGGT	AGCGGTGGCG	GTGGTAGCGG	TGGCGGTGGC	AGCGGTGGTG
401	GTGGCTCTGG	TGGCGGTGGC	TCTGAAGTGC	AGCTGCAGCA	GTCCGGTCCG
451	GAGCTCGTTG	AACCGGGCGC	TTCTGTGAAA	CTGTCTTGCA	CTGCATCTGG
501	TTTCAACATT	AAGGACACCT	ACATGCACATG	GGTGAACAA	CGCCCGGAAC
551	AGGGTCTGGA	GTGGATCGGT	CGCATCGATC	CGGCTAACGG	TACAGCAAA
601	TACGTGCCAA	AATTCAGGG	TAAAGCAACC	ATCACTGCTG	ATACCTCCTC
651	TAACACTGCT	TACCTGCAGC	TGACTTCCCT	GACTAGCGAA	GACACCGCGG
701	TTTATTACTG	CGCTCCGTTT	GGCTACTATG	TCAGCGATTA	CGCAATGGCC
751	TACTGGGGTG	AGGGCACCTC	TGTTACCGTT	TCTAGCACAC	CGGTGTGAGA
801	AAAACAGCTG	CGCGAGGTGG	TCGCGAATAC	GATTACCCCG	CTGATGGCGG
851	CCCAGTCTGT	TCCAGGCATG	GCGGTGGCCG	TTATTTATCA	GGGAAAACCG
901	CACATATTACA	CATTTGGCAA	GGCCGATATC	GCGGCGAATA	AACCCGTTAC
951	GCCTCAGACC	CTGTTTCGAGC	TGGGTTCTAT	AAGTAAAACC	TTACCCGCGG
1001	TTTTAGGTGG	GGATGCCATT	GCTCGCGGTG	AAATTTGCTG	GGACGATGCG
1051	GTGACCAGAT	ACTGGCCACA	GCTGACGGGC	AAGCAGTGGC	AGGGTATTTCG
1101	TATGCTGGAT	CTCGCCACCT	ACACCGCTGG	CGGCCCTGCCG	CTACAGGTAC
1151	CGGATGAGGT	CACGGATAAC	GCCTCCCTGC	TGCGCTTTTA	TCAAACTGG
1201	CAGCCGCAGT	GGAGCCCTGG	CACAACGCGT	CTTTACGCCA	ACGCCAGCAT
1251	CGGCTCTTTT	GTCGCGCTGG	CGGTCAAACC	TTCTGGCATG	CCCTATGAGC
1301	AGGCCATGAC	GACGCGGGTC	CTTAAGCCGC	TCAAGCTGGA	CCATACCTGG
1351	ATTAACGTGC	CGAAAGCGGA	AGAGGCGCAT	TACGCCCTGGG	GCTATCGTGA
1401	CGGTAAAGCG	GTGCGCGTTT	CGCCGGGTAT	GCTGGATGCA	CAAGCCTATG
1451	CGGTGAAAAC	CAACGTGCAG	GATATGGCGA	ACTGGGTCTAT	GGCAACATG
1501	GCGCCGGAGA	ACGTTGCTGA	TGCCTCACTT	AAGCAGGGCA	TCGGCTGGGC
1551	GCAGTCGCGC	TACTGGCGTA	TCGGGTCAAT	GTATCAGGGT	CTGGGCTGGG
1601	AGATGTCTAA	CTGGCCCGTG	GAGGCCAACA	CGGTGGTCTGA	GACGAGTTTTT
1651	GGTAATGTAG	CACTGGCGCC	GTTGCCCGTG	GCAGAAGTGA	ATCCACCGGC
1701	TCCCCCGGTC	AAAGCGTCCT	GGGTCCATAA	AACGGGCTCT	ACTGGCGGGT
1751	TTGGCGCGTA	CGTGGCCTTT	ATTCCTGAAA	AGCAGATCGG	TATTGTGATG
1801	CTCGGAATA	CAAGCTATCC	GAACCCGGCA	CGCGTTGAGG	CGGCATACCA
1851	TATCCTCGAG	GCGCTACAG			

Figure 4E

1 AGGAATTATC ATATGAAATA CCTGCTGCCG ACCGCTGCTG
CTGGTCTGCT
51 GCTCCTCGCT GCCCAGCCGG CCATGGCCGA CATCGTCTGT
ACCCAGAGCC
101 CGGCAAGCCT GTCTGTTTCC CTGGGCCAGC GTGCCACTAT
GTCCTGCAGA
151 GCGGGTGAGT CTGTTGACAT TTTCGGTGTC GGTTTTCTGC
ACTGGTACCA
201 ACAGAAACCG GGTCAGCCGC CAAACTGCT GATCTATCGT
GCTTCTAAC
251 TGGAGTCCGG CATCCCGGTA CGTTTCTCCG GTAGTGCTC
TGGTACTGAT
301 TTTACCTGA TTATCGACCC GGTGAAGCA GACGATGTTG
CCACCTACTA
351 TTGCCAGCAG ACCAACGAGG ATCCGTACAC CTTGCGTGCG
GGTACTAAAC
401 TGGAGATCAA AGGCGGTGGT GGTCTGTGTG GTGGTGGTAG
CGGTGGCGGT
451 GGTAGCGGTG GCGGTGGCAG CGGTGGTGGT GGCTCTGGT
GCGGTGGCTC
501 TGAAGTGCA CTGCAGCAGT CCGTGCGGA GCTCGTTGAA
CCGGGCGCTT
551 CTGTGAAACT GTCTTGCACT GCATCTGGTT TCAACATTAA
GGACACCTAC
601 ATGCACTGGG TGAACAACG CCCGGAACAG GGTCTGGAGT
GGATCGGTCG
651 CATCGATCCG GCTAACGGTA ACAGCAAATA CGTGCCAAAA
TTCCAGGGTA
701 AAGCAACCAT CACTGCTGAT ACCTCCTCTA ACATGCTTAA
CCTGCAGCTG
751 ACTTCCCTGA CTAGCGAAGA CACCGCGGTT TATTACTGCG
CTCCGTTCCG
801 CTAATATGTC AGCGATTACG CAATGGCCTA CTGGGGTCAG
GGCACCTCTG
851 TTACCGTTTC TAGCACACCG GTGTCAGAAA AACAGCTGGC
GGAGGTGGTC
901 GCGAATACGA TTACCCCGCT GATGGCGGCC CAGTCTGTT
CAGGCATGGC
951 GGTGGCCGTT ATTTATCAGG GAAACCGCA CTATTACACA
TTTGCAAGG
1001 CCGATATCGC GGCGAATAAA CCCGTTACGC CTCAGACCCT
GTTTCGAGCTG

1051 GGTTCATATAA GTAAACCTT CACCGGCGTT TTAGTGGGG
ATGCCATTGC
1101 TCGCGGTGAA ATTTTCGTGG ACGATGCGGT GACCAGATAC
TGGCCACAGC
1151 TGACGGGCAA GCAGTGGCAG GGTATTTCGTA TGCTGGATCT
CGCCACCTAC
1201 ACCGCTGGCG GCCTGCCGCT ACAGGTACCG GATGAGGTCA
CGGATAACGC
1251 CTCCTGCTG CGCTTTTATC AAACTGGCA GCCGCAGTGG
AAGCCTGGCA
1301 CAACGCGTCT TTACGCCAAC GCCAGCATCG GTCTTTTTGG
TGCGCTGGCG
1351 GTCAAACCTT CTGGCATGCC CTATGAGCAG GCCATGACGA
CGCGGGTCCT
1401 TAAGCCGCTC AAGCTGGACC ATACCTGGAT TAACGTGCCG
AAAGCGGAAG
1451 AGGCGCATTA CGCCTGGGGC TATCGTGACG GTAAAGCGGT
GCGCGTTTCG
1501 CCGGGTATGC TGGATGCACA AGCCTATGGC GTGAAAACCA
ACGTGCAGGA
1551 TATGGCGAAC TGGGTATGG CAAACATGGC GCCGAGAAC
GTTGCTGATG
1601 CCTCACTTAA GCAGGGCATC GCGCTGGCGC AGTCGCGTA
CTGGCGTATC
1651 GGGTCAATGT ATCAGGGTCT GGGCTGGGAG ATGCTCAACT
GGCCCCGTGA
1701 GGCCAACACG GTGGTCGAGA CGAGTTTGG TAATGTAGCA
CTGGCGCCGT
1751 TGCCCGTGGC AGAAGTGAAT CCACCGCTC CCCCAGTCAA
AGCGTCCTGG
1801 GTCCATAAAA CGGGCTCTAC TGGCGGGTTT GGCGCGTACG
TGGCCTTTAT
1851 TCCTGAAAAG CAGATCGGTA TTGTGATGCT CGCGAATACA
AGCTATCCGA
1901 ACCCGGCACG CGTTGAGGCG GCATACCATA TCCTCGAGGC
GCTACAGTAG
1951 GAATTCGAGC TCCGTCGACA AGCTTGGCGC CGCACTCGAG
ATCAAACGGG
2001 CTAGCCAGCC AGAACTCGCC CCGGAAGACC CCGAGGATGT
CGAGCACCA
2051 CACCACCACC ACTGAGATCC GGCTGCTAAC AAAGCCCGAA
AGGAAGCTGA
2101 GTTGGCTGCT GCCACCGCTG AGCAATAACT AGCATAACCC
CTTGGGGCCT
2151 CTAAACGGGT CTTGAGGGGT TTTTGTGTA AAGGAGGAAC
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2201 TTGGCGAATG GGACGCGCCC TGTAGCGGCG CATTAAAGCGC
GGCGGGTGTG
2251 GTGGTTACGC GCAGCGTGAC CGCTACACTT GCCAGCGCCC
TAGCGCCCCG
2301 TCCTTTCGCT TTCTTCCCTT CCTTCTCGC CACGTTTCGC
GGCTTTCCCC
2351 GTCAAGCTCT AAATCGGGG CTCCCTTAG GTTCCGATT
TAGTGCTTTA
2401 CGGCACCTCG ACCCAAAAA ACTTGATTAG GGTGATGGTT
CACGTAGTGG
2451 GCCATCGCCC TGATAGACGG TTTTTCGCCC TTTGACGTG
GAGTCCACGT
2501 TCTTTAATAG TGGACTCTTG TTCCAAACTG GAACAACACT
CAACCTATC
2551 TCGGTCTATT CTTTGTATT ATAAGGGATT TTGCCGATT
CGGCCTATTG
2601 GTTAAAAAAT GAGCTGATT AACAAAAATT TAACGCGAAT
TTTAACAAAA
2651 TATTAACGCT TACAATTTCC TGATGCGGTA TTTTCTCCTT
ACGCATCTGT
2701 GCGGTATTTC ACACCGCATA TGGTGCACTC TCAGTACAA
CTGCTCTGAT
2751 GCCGCATAGT TAAGCCAGCC CCGACACCCG CCAACACCCG
CTGACGCGCC
2801 CTGACGGGCT TGTCTGCTCC CGGCATCCGC TTACAGACAA
GCTGTGACCG
2851 TCTCCGGGAG CTGCATGTGT CAGAGGTTTT CACCGTCATC
ACCGAAACGC
2901 GCGAGACGAA AGGGCCTCGT GATACGCCTA TTTTATAGG
TTAATGTCAT
2951 GATAATAATG GTTCTTAGA CGTCAGGTGG CACTTTTCGG
GGAAATGTGC
3001 GCGGAACCCC TATTTGTTA TTTTCTAAA TACATTCAAA
TATGTATCCG
3051 CTCATGAGAC AATAACCCTG TGGCAGCATC ACCCGACGCA
CTTTGCGCCG
3101 AATAAATACC TGTGACGGAA GATCACTTCG CAGAATAAAT
AAATCTGGT
3151 GTCCTGTG ATACCGGGAA GCCCTGGGCC AACTTTTGGC
GAAATGAGA
3201 CGTTGATCGG CACGTAAGAG GTTCCAACCT TCACCATAAT
GAAATAAGAT
3251 CACTACCGGG CGTATTTTTT GAGTTATCGA GATTTCAGG
AGCTAAGGAA
3301 GCTAAAATGG AGAAAAAAT CACTGGATAT ACCACCGTTG
ATATATCCCA

3351 ATGGCATCGT AAAGAACATT TTGAGGCATT TCAGTCAGTT
GCTCAATGTA
3401 CCTATAACCA GACCGTTCAG CTGGATATTA CGGCCTTTTT
AAAGACCGTA
3451 AAGAAAAATA AGCACAAGTT TTATCCGGCC TTTATTACA
TTCTTGCCCC
3501 CCTGATGAAT GCTCATCCGG AATTCGGTAT GGCAATGAA
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3551 TGGTGATATG GGATAGTGTT CACCCTTGTT ACACCGTTTT
CCATGAGCAA
3601 ACTGAAACGT TTTCATCGCT CTGGAGTGAA TACCACGACG
ATTTCCGGCA
3651 GTTTCTACAC ATATATTCGC AAGATGTGGC GTGTTACGGT
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3701 CCTATTTCCT TAAAGGGTTT ATTGAGAATA TGTTTTTCGT
CTCAGCCAAT
3751 CCCTGGGTGA GTTTCACCAG TTTTGATTTA AACGTGGCCA
ATATGGACAA
3801 CTTCTTCGCC CCCGTTTCA CGATGGGCAA ATATTATACG
CAAGGCGACA
3851 AGGTGCTGAT GCCGCTGGCG ATTCAGGTTC ATCATGCCGT
CTGTGATGGC
3901 TTCCATGTCG GCAGAATGCT TAATGAATTA CAACAGTACT
GCGATGAGTG
3951 GCAGGGCGGG GCGTAAAGAC AGATCGCTGA GATAGGTGCC
TCACTGATTA
4001 AGCATTGGTA ACTGTCAGAC CAAGTTTACT CATATATACT
TTAGATTGAT
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4101 TAATCTCATG ACCAAATCC CTTAACGTGA GTTTTCGTTT
CACTGAGCGT
4151 CAGACCCCGT AGAAAAGATC AAAGGATCTT CTTGAGATCC
TTTTTTCTG
4201 CGCGTAATCT GCTGCTTGCA AACAAAAAAA CCACCGCTAC
CAGCGTGGT
4251 TTGTTGCCG GATCAAGAGC TACCAACTCT TTTTCCGAAG
GTAAGTGGCT
4301 TCAGCAGAGC GCAGATACCA AATACTGTTT TTCTAGTGTA
GCCGTAGTTA
4351 GGCCACCACT TCAAGAACTC TGTAGCACCG CCTACATACC
TCGCTCTGCT
4401 AATCCTGTTA CCAGTGGCTG CTGCCAGTGG CGATAAGTCG
TGTCTTACCG
4451 GGTGGACTC AAGACGATAG TTACCGGATA AGGCGCAGCG
GTCGGGCTGA

4501 ACGGGGGGTT CGTGACACACA GCCCAGCTTG GAGCGAACGA
 CCTACACCGA
 4551 ACTGAGATAC CTACAGCGTG AGCTATGAGA AAGCGCCACG
 CTTCCCGAAG
 4601 GGAGAAAGGC GGACAGGTAT CCGGTAAGCG GCAGGGTCGG
 AACAGGAGAG
 4651 CGCAGAGGG AGCTTCCAGG GGGAAACGCC TGGTATCTTT
 ATAGTCCTGT
 4701 CGGGTTTCGC CACCTCTGAC TTGAGCGTCG ATTTTGTGA
 TGCTCGTCAG
 4751 GGGGGCGGAG CCTATGAAA AACGCCAGCA ACGCGGCCTT
 TTTACGGTTC
 4801 CTGGCCTTTT GCTGGCCTTT TGCTCACATG TTCTTTCCTG
 CGTTATCCCC
 4851 TGATTCTGTG GATAACCGTA TTACCGCCTT TGAGTGAGCT
 GATACCGCTC
 4901 GCCGCAGCCG AACGACCGAG CGCAGCGAGT CAGTGAGCGA
 GGAAGCGGAA
 4951 GAGCGCCCAA TACGCAAACC GCCTCTCCCC GCGCGTTGGC
 CGATTCATTA
 5001 ATGAGCTGG CACGACAGGT TTCCCGACTG GAAAGCGGGC
 AGTGAGCGCA
 5051 ACGCAATTAA TGTGAGTTAG CTCACTCATT AGGCACCCCA
 GGCTTTACAC
 5101 TTTATGCTTC CGGCTCGTAT GTTGTGTGGA ATTGTGAGCG
 GATAACAATT
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 TTTAGGTGAC
 5201 ACTATAGAAT ACTCAAGCTT TCTAGATTAA GG

Figure 6

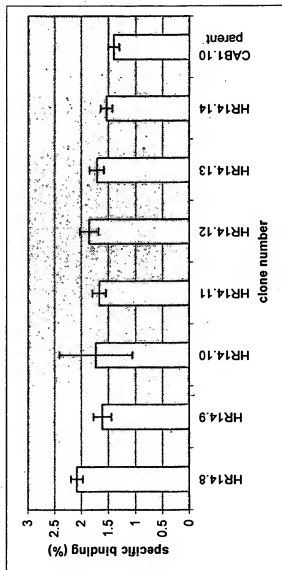


Figure 7

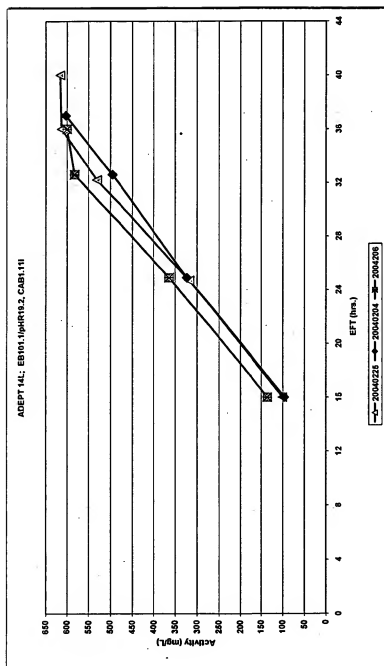


Figure 8

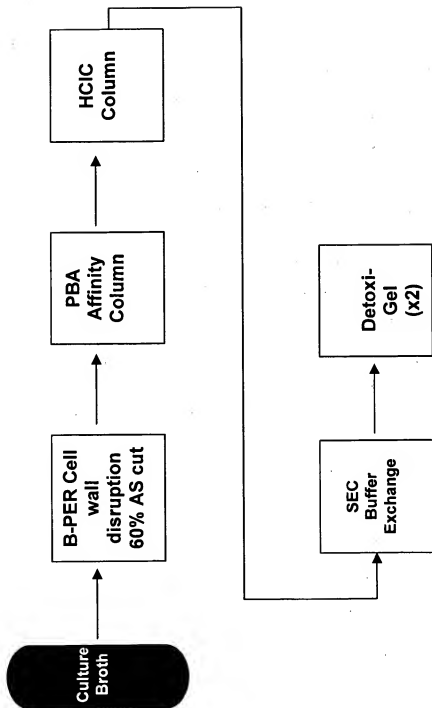


Figure 9

Lane 1: molecular weight standard; Lanes 3-5: unrelated proteins; lane 6: CAB1.11i.



Figure 10A

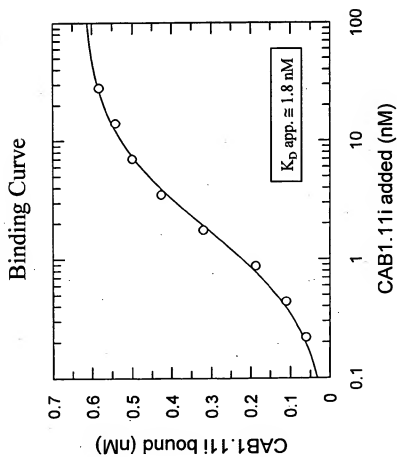


Figure 10B

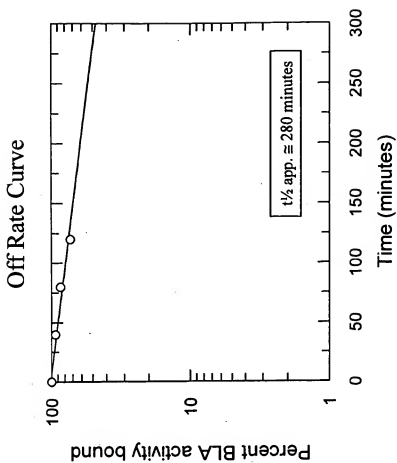


Figure 5

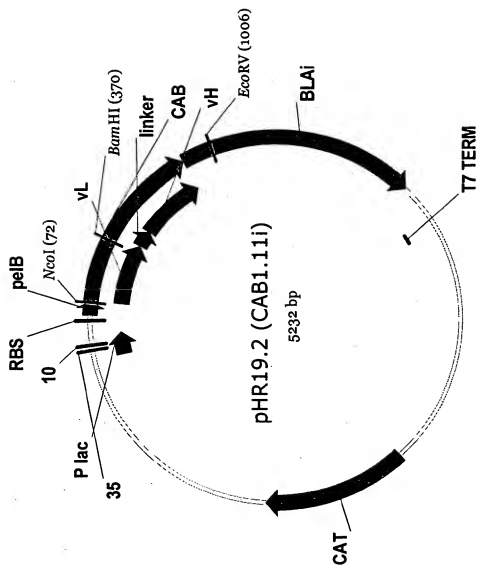


Figure 11

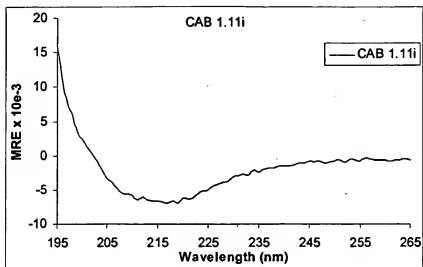


Figure 12

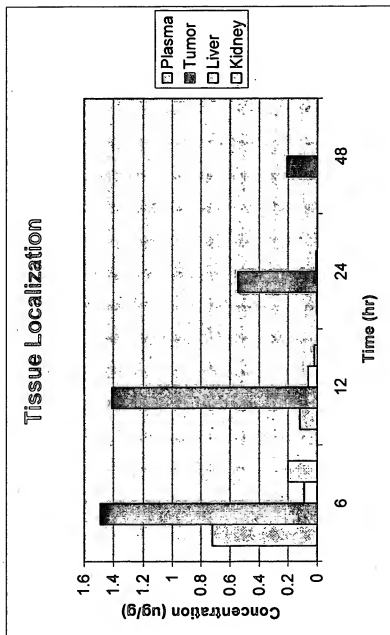


Figure 13

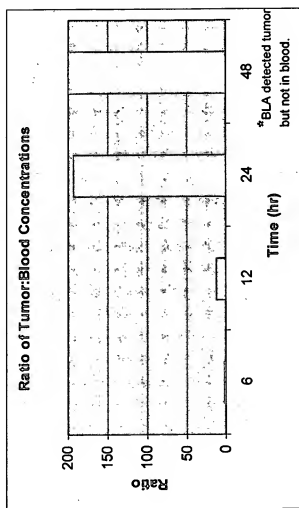


Figure 14

